



AGRICULTURAL RESEARCH INSTITUTE

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# THE JOURNAL OF BIOLOGICAL CHEMISTRY

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MEMORIAL FUND

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VOLUME 177

~~BALTIMORE~~

1949

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THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC.

PUBLISHED AT YALE UNIVERSITY FOR  
THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC.  
WAVERLY PRESS, INC.  
BALTIMORE 2, U. S. A.

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### CORRECTION

On page 479, lines 10 to 13 of the table and line 13 of the text, Vol. 175, No. 1, August, 1948, in each instance read *per 1 gm.* for *per 100 gm.*



# PARALYTIC SHELLFISH POISON

## IV. BASES ACCOMPANYING THE POISON\*

By BYRON RIEGEL, D. WARREN STANGER, DONALD M. WIKHOLM,  
JAMES D. MOLD, AND HERMANN SOMMER

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Williams Hooper Foundation, University of California, San Francisco)

(Received for publication, May 29, 1948)

Mussel poison extracts, even after preliminary purification (1, 2), retain varying amounts of other bases. A study of these bases was made for the following reasons. First, the fact that these bases contaminate the poison in partially purified extracts indicates that they are similar in chemical properties to the poison. Second, the removal of these bases would be made easier if they were identified.

Ackermann isolated and identified several nitrogenous bases from the extract of whole mussels. In *Mytilus edulis* L. he found adenine, arginine, betaine, neosine,<sup>1</sup> methylpyridinium hydroxide, and crangonine (4), and in *Arca noae*, arcaine (1,4-diguanidobutane),  $\gamma$ -butyrobetaine, stachydrine, homarine, and carnitine (5, 6). Betaine has been identified (7) and taurine has been reported (8) in liver extracts from *Mytilus californianus* Conrad. In the present investigation taurine was isolated and identified.

Betaine was identified in the filtrates from the ion exchange of crude, mussel liver extracts on Decalso and in the acid filtrates from the chromatographic fractionation of the crude extracts on norit A. The fact that no betaine was found in Decalso eluates indicates that it is not adsorbed by Decalso.

Choline was found in Decalso eluates; after chromatography of the eluates on norit A, it was found in the primary acid filtrates. The same behavior of choline was observed when crude, mussel liver extracts were chromatographed.

A third base, homarine (methylbetaine of picolinic acid), was identified in the acidified methanol eluates from the chromatographic fractionation of Decalso eluates or of crude, mussel liver extracts.

The behavior of the bases accompanying mussel poison is summarized in Diagrams 1 and 2. It is interesting to note that these three bases are

\* The work described in this paper was done under a contract between the Chemical Corps, Camp Detrick, Frederick, Maryland, and Northwestern University and the University of California.

<sup>1</sup> Strack *et al.* (3) showed that "neosine" chloroaurate actually consisted of mixed crystals of betaine and choline chloroaurate.

similar in chemical structure; each contains quaternary nitrogen. This fact suggests that mussel poison may also be a quaternary base.

No systematic attempt was made to isolate free amino acids from mussel liver extracts. However, the precipitate from concentrated ion exchange filtrates at pH 5.0 was identified as tyrosine.

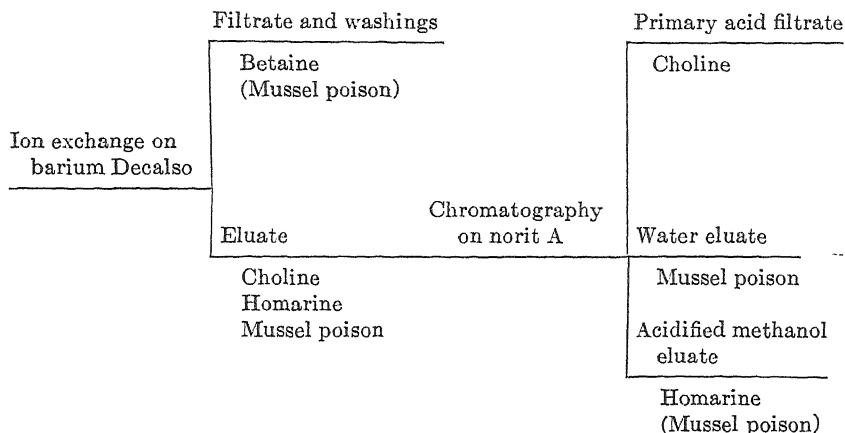


DIAGRAM 1. Separation of the bases in crude liver extract from poison mussels by ion exchange on Decalso followed by chromatography of the poison concentrate on acid-washed norit A.

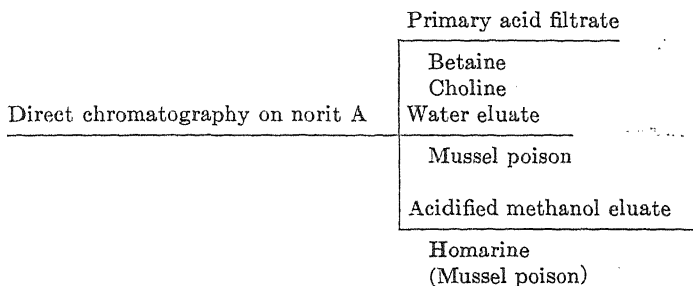


DIAGRAM 2. Separation of the bases in crude liver extract from poison mussels by chromatography on acid-washed norit A.

At least one other substance, probably a base, was found associated with the purified mussel poison obtained by chromatography. Its presence was established by the ultraviolet absorption spectrum of the partially purified poison. This showed a maximum at 2575 Å, which was at first thought to be due to the poison itself. However, after further purification of the

poison by precipitation as the rufanate or helianthate, no absorption in this region could be detected. The ultraviolet-absorbing material was found concentrated in the mother liquors. Although the amount of material available was insufficient for identification, its absorption maximum showed it to be different from homarine (maximum, 2730 Å).

#### EXPERIMENTAL<sup>2, 3</sup>

*Identification of Taurine in Alcohol-Insoluble Fraction of Decolorized Mussel Liver Extracts*—25 gm. of the alcohol-insoluble fraction of a decolorized liver extract (1) were dissolved in 70 ml. of boiling water, treated with 1.0 gm. of norit A, and filtered. Dilution of the hot filtrate with 280 ml. of ethanol and cooling furnished white crystals and some amorphous yellow material. Crystallization from 20 per cent aqueous alcohol yielded 5.2 gm. of white needles which gave a qualitative test for sulfur. The material was identified as taurine by elementary analysis.

$C_2H_7NO_3S$ . Calculated. C 19.19, H 5.64, N 11.19  
Found. " 19.07, " 5.52, " 11.05

*Identification of Bases Found in Acid Filtrate and Acidified Methanol Eluate after Chromatography of Crude Mussel Liver Extracts on Norit A*—The acid filtrates from several chromatographic fractionations of crude liver extracts were combined (3675 ml., total solids, 107.4 gm.). The bases were precipitated as the reineckates by the addition of 2500 ml. of freshly prepared, saturated, aqueous Reinecke salt solution. The precipitate, which had a glistening appearance characteristic of choline reineckate, was collected, washed with cold water, and dried (45.1 gm.). A 5 gm. portion of the reineckate, suspended in 45 ml. of water, was treated with 25 ml. of concentrated  $NH_4OH$  to dissolve the reineckates of those bases containing an acidic group (9). The mixture was stirred for 30 minutes and then centrifuged. The residue was extracted twice more with dilute  $NH_4OH$ , and the final residue was suspended in a little water, collected, and dried (1.65 gm.). The combined ammoniacal solutions were acidified with concentrated HCl and the precipitate was collected, washed, and dried (3.35 gm., m.p. 149–151°).

The combined, acidified, methanol eluates from several chromatographic fractionations (total solids, 20.9 gm.) were concentrated to 200 ml., diluted to 1.0 liter with water, and concentrated to 400 ml. to remove most of the methanol. A 10 ml. portion of the concentrate was made alkaline with

<sup>2</sup> All melting points were taken with a Fisher-Johns melting point apparatus and are corrected.

<sup>3</sup> The microanalyses were done by June Anderson, Patricia Craig, Margaret Ledyard, and Rita Pivan, Northwestern University.

concentrated  $\text{NH}_4\text{OH}$  and treated with 10 ml. of the Reinecke salt solution, but no precipitate was obtained. After 10 minutes the solution was acidified, and a precipitate formed immediately, which indicated that bases with an acidic group were present. The remainder of the extract was treated with saturated Reinecke salt solution without the addition of  $\text{NH}_4\text{OH}$ . The red precipitate was collected and dried (10.7 gm.).

*Identification of Betaine*—The alkali-soluble reineckate (3.35 gm.) was dissolved in 150 ml. of a 1:1 acetone-water mixture and treated with 218 ml. of a 0.6 per cent  $\text{Ag}_2\text{SO}_4$  solution (10). After removal of the silver reineckate, the filtrate was treated with an equivalent amount of  $\text{BaCl}_2$  solution. The  $\text{BaSO}_4$  was collected and the filtrate concentrated to 10 ml. One-third of the filtrate was treated with gold chloride solution, and the chloroaurate was crystallized twice from 1 per cent  $\text{HCl}$  (m.p.  $229-230^\circ$  with decomposition). This was identified as betaine chloroaurate.

$\text{C}_5\text{H}_{11}\text{NO}_2 \cdot \text{HAuCl}_4$ . Calculated, Au 43.14; found, Au 42.92

The remaining two-thirds of the base chloride was converted to the picrate (m.p.  $181-183^\circ$ ). A mixture with an authentic sample of betaine picrate showed no depression of the melting point.

A portion of the purified picrate was converted to the chloride. This on crystallization from 95 per cent alcohol melted at  $243-246^\circ$  with decomposition. A mixture with an authentic sample of betaine chloride (m.p.  $243-246^\circ$  with decomposition) showed no depression of the melting point.

$\text{C}_5\text{H}_{11}\text{NO}_2 \cdot \text{HCl}$ . Calculated, N 9.12; found, N 9.33

*Identification of Choline*—The alkali-insoluble reineckate (1.65 gm.) was dissolved in 50 ml. of the aqueous acetone and converted to the chloride.

The chloroaurate was prepared from the chloride and after three crystallizations from water melted at  $245-248^\circ$  with decomposition. A mixture with choline chloroaurate (m.p.  $244-245^\circ$  with decomposition) melted at  $246-247^\circ$  with decomposition.

$\text{C}_5\text{H}_{14}\text{NO} \cdot \text{AuCl}_4$ . Calculated, Au 44.50; found, Au 44.57

*Identification of Homarine*—The reineckate obtained from the acidified methanol eluates (10.7 gm.) was converted to the chloride. A portion of the chloride was converted to the picrate (m.p.  $159-161^\circ$ ). The melting point of the mixture with homarine picrate (m.p.  $159-161^\circ$ ) showed no depression. The remainder of the chloride was converted to the chloroplatinate (m.p.  $191-193^\circ$  with decomposition).

$(\text{C}_7\text{H}_7\text{NO}_2)_2 \cdot \text{H}_2\text{PtCl}_6$ . Calculated, Pt 28.53; found, Pt 28.16

*Identification of Bases Found in Filtrate and Eluate after Ion Exchange of Crude, Mussel Liver Extracts on Barium Decalco; Identification of Betaine*

*in Filtrate*—The filtrate and washings from an ion exchange preparation on barium Decalso were concentrated and made alkaline with  $\text{NH}_4\text{OH}$ . Treatment with a freshly prepared saturated solution of Reinecke salt gave no immediate precipitate. The solution was acidified with  $\text{HCl}$ , and the reineckate which precipitated was converted to the chloride. The base was identified as betaine from the picrate (m.p.  $181\text{--}183^\circ$ ) and the analysis of the chloride.

$\text{C}_5\text{H}_{11}\text{NO}_2 \cdot \text{HCl}$ . Calculated, N 9.12; found, N 9.27

*Identification of Choline in Eluate*—An eluate obtained from an ion exchange preparation on barium Decalso was dissolved in a saturated, aqueous solution of ammonium reineckate diluted with an equal volume of acetone. The solution was concentrated to half volume and the insoluble reineckate collected. All of the poison remained in the filtrate. The reineckate was decomposed and the chloroaurate prepared (m.p.  $245\text{--}247^\circ$  with decomposition). The mixture with choline chloroaurate (m.p.  $246\text{--}248^\circ$  with decomposition) melted at  $245\text{--}248^\circ$  with decomposition.

$\text{C}_5\text{H}_{14}\text{NO} \cdot \text{AuCl}_4$ . Calculated, Au 44.50; found, Au 44.47

*Identification of Bases Found in Acid Filtrate and Acidified Methanol Eluate after Chromatography of Decalso Eluate on Norit A; Identification of Choline in Acid Filtrate*—The primary acid filtrate obtained by chromatography of a Decalso eluate on norit A was concentrated to a small volume. The precipitate, which formed when the solution was made alkaline with  $\text{NH}_4\text{OH}$  and treated with Reinecke salt, was collected and dried. Acidification of the filtrate with  $\text{HCl}$  gave no precipitate, which indicated that no base of the betaine type was present. The reineckate was converted to the chloride and that in turn to the picrate and chloroaurate. The picrate (m.p.  $245\text{--}247^\circ$  with decomposition), when mixed with choline picrate (m.p.  $245\text{--}247^\circ$  with decomposition), showed no depression in melting point. The analysis of the chloroaurate confirmed the identification of choline.

$\text{C}_5\text{H}_{14}\text{NO} \cdot \text{AuCl}_4$ . Calculated, Au 44.50; found, Au 44.76

*Identification of Homarine in Acidified Methanol Eluate*—The acidified methanol eluate obtained by chromatography of a Decalso eluate on norit A was evaporated and the aqueous solution of the residue was treated with Reinecke salt. The reineckate which formed was converted to the chloride and the chloride to the picrate (m.p.  $158\text{--}160^\circ$ ). The mixture with homarine picrate (m.p.  $159\text{--}161^\circ$ ) melted at  $158\text{--}160^\circ$ .

*Identification of Tyrosine in Filtrate after Ion Exchange of Mussel Liver Extracts*—The filtrate from an ion exchange preparation was concentrated and the copious, flocculent mass of crystals which separated at pH 5.0 was collected and washed with cold water. After two crystallizations from hot

water, the material was dissolved in 0.1 N NaOH and centrifuged. The supernatant was decanted and neutralized with HCl. The precipitate was washed with water, dissolved in 0.1 N HCl, and centrifuged. The supernatant was decanted, neutralized with NaOH, and the precipitate was washed with water, alcohol, and ether and dried. The material was identified as tyrosine by strongly positive ninhydrin and Millon tests and by elementary analysis.

$C_9H_{11}NO_3$ . Calculated. C 59.66, H 6.12, N 7.73  
Found. " 59.24, " 6.30, " 7.49, ash 0.5

#### SUMMARY

1. Betaine, choline, homarine, taurine, and tyrosine have been isolated and identified in liver extracts from poison mussels.
2. Choline and homarine, both quaternary bases, remain closely associated with mussel poison through the preliminary steps in its purification.
3. An additional base has been detected by its characteristic ultraviolet absorption spectrum but has not been identified.

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## PARALYTIC SHELLFISH POISON

### V. THE PRIMARY SOURCE OF THE POISON, THE MARINE PLANKTON ORGANISM, *GONYAULAX CATENELLA*\* -

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(Received for publication, May 29, 1948)

Shellfish, such as clams, mussels, and oysters, feed on marine plankton. At least one species of dinoflagellate, *Gonyaulax catenella* Whedon and Kofoid, has been shown by Sommer and coworkers (1) to be a source of the poison found in the California mussel, *Mytilus californianus* Conrad. The present investigation was carried out to determine the feasibility of collecting the plankton and extracting and concentrating the poison from this source for chemical studies.

The dinoflagellate, *Gonyaulax catenella*, is too small to be visible to the naked eye. However, if the concentration of these organisms, which multiply very rapidly under favorable conditions, approaches 1,000,000 per liter, a reddish brown coloration of the surface water is observed. When the concentration reaches 20 to 40 million cells per liter, the ocean is a deep rust-red by day and brilliantly luminescent by night. High concentrations of certain non-poisonous marine plankton organisms may also appear as "red water."

For the large scale collection, the United States Navy<sup>1</sup> supplied a patrol boat equipped with two Sharples supercentrifuges adapted as clarifiers. As red water is easily visible from the air, the Naval Lighter-Than-Air Command patrolled several miles of the coastline of California to find the area of its greatest concentration. The blimp directed the boat by radio contact to a spot in Monterey Bay just south of Santa Cruz and dropped smoke flares to locate the exact position of the red water. The boat was allowed to drift into the colored areas, and the surface water of the bay was pumped through the centrifuges. In this way the plankton was removed from 5000 liters of sea water in a period of 3 hours.

The plankton, which consisted mainly of *Gonyaulax catenella*, was de-

\* The work described in this paper was done under a contract between the Chemical Corps, Camp Detrick, Frederick, Maryland, and Northwestern University and the University of California.

<sup>1</sup> The invaluable assistance volunteered by several branches of the United States Navy made this investigation possible.

posited as a reddish brown residue in the bowls of the centrifuges. This residue was suspended in 95 per cent ethanol containing 4 ml. of concentrated HCl per liter. Bioassay of the suspension showed 500,000 mouse units of poison<sup>2,3</sup> (total solids, 40.6 gm.). The first extract of the poison was obtained by centrifuging. The residue was resuspended in additional acidified ethanol, stirred for 5 hours, and centrifuged. This process was repeated three times, once in the cold and twice at 70° (Table I).

The combined extract, after decolorization with Nuchar C and removal of the alcohol, was purified by chromatographic fractionation on acid-washed norit A (2). The fraction which contained the poison was rechromatographed on a column of fresh carbon. The best fraction from the

TABLE I  
*Extraction of Poison from Collection of Marine Plankton*

Extract No.	Volume	Poison	Total solids	Toxicity
	<i>ml.</i>	<i>mouse units</i>	<i>gm.</i>	<i>mouse units per mg.</i>
1	1720	236,000	17.55	13.4
2	315	16,900		
3	445	12,800	0.75	17.1
4	435	8,390	1.04	8.06
5	490	<9,760		
Total.....	3405	274,000		
Combined extract reassayed	3405	285,000	18.1	15.8
Residue after extraction			22.5	

third chromatogram contained 31,000 mouse units and had a toxicity of 1.65 mouse units per microgram (Table II). Only under the most favorable conditions would this method of collection furnish enough material for chemical study of the poison.

Although marine plankton organisms serve as the primary source of food for all marine life, their chemistry has not been studied extensively. Am-

<sup>2</sup> 1 mouse unit is that quantity of poison which, on intraperitoneal injection, will kill a 20 gm. mouse in 15 minutes.

<sup>3</sup> A second collecting expedition was made 4 days later, but the red water had been completely dissipated. Although the centrifuges were run for 12 hours over a 2 day period, less than 30,000 mouse units of poison (total solids, 41.84 gm.) were obtained. The collection consisted mainly of two species of *Ceratium*, but included other peridinians as well. It was suspended in acidified ethanol, extracted, and the combined extract chromatographed on acid-washed norit A. The fractions obtained were combined with the material from the first collection and used for the study of the bases found in marine plankton.

monia, methylamine, and trimethylamine have been isolated from fresh-water and sea water plants and fresh-water plankton (3). In the present investigation, a study was made of the bases present in marine plankton rich in *Gonyaulax catenella*. Non-toxic fractions from all of the chromatograms were combined and used in this study.<sup>3</sup> Trimethylamine and choline were identified in the primary acid filtrate. A small amount of a base of the betaine type was isolated but not identified. It was shown to be neither betaine nor homarine.

The acidified methanol eluate was also found to contain a base of the betaine type, but insufficient material prevented isolation and identification.

TABLE II  
*Chromatographic Fractionation of Poison from Marine Plankton*

Toxic fractions	Weight of acid-washed norit A	Poison	Total solids	Toxicity
	gm.	mouse units	gm.	mouse units per mg.
Starting material		289,000	13.2	21.9
1st fractionation	15	259,000	7.87	32.9
2nd     "	15	118,000	1.25	94.4
3rd     "	6			
1		35,000	0.067	522
2		37,000	0.036	1030
3		31,400	0.019	1650

The identification of choline in the plankton, as well as in mussel liver extracts (4), indicates that the plankton may be the source of the choline found in the mussel liver. No attempt has been made to isolate trimethylamine from mussel liver extracts, although it may be present, since it is found in the plankton on which the mussel feeds.

#### EXPERIMENTAL<sup>4, 5</sup>

*Chromatographic Fractionation on Norit A of Poison Extract from Collection of Marine Plankton*—The combined extract (3405 ml., 285,000 mouse units) was decolorized by shaking with 10 gm. of Nuchar C and filtering through an additional 20 gm. of the carbon on a Büchner funnel. The colorless filtrate (3480 ml., 319,000 mouse units) was concentrated to remove the alcohol. The solution (100 ml., 0.98 N HCl, 289,000 mouse units) was chromatographed on acid-washed norit A in the usual manner (2).

<sup>4</sup> All melting points were taken with a Fisher-Johns melting point apparatus and are corrected.

<sup>5</sup> The microanalyses were done by Patricia Craig, Northwestern University.

*Identification of Bases Found in Acid Filtrate from Chromatography of Poison Extract on Norit A*—The acid filtrates from the three chromatographic fractionations were combined and made alkaline with  $\text{NH}_4\text{OH}$ . Treatment with Reinecke salt furnished 0.832 gm. of alkali-insoluble reineckate. Acidification of the filtrate with  $\text{HCl}$  furnished 0.594 gm. of alkali-soluble reineckate.

*Identification of Trimethylamine*—The alkali-insoluble reineckate was dissolved in 50 ml. of a 1:1 acetone-water solution and converted to the chloride by treatment with equivalent amounts of  $\text{Ag}_2\text{SO}_4$  and  $\text{BaCl}_2$ . A few drops of the solution were treated with 2 ml. of 20 per cent  $\text{KOH}$  solution. The characteristic odor of trimethylamine was noted. The remainder of the solution was evaporated in a flask containing 1.0 gm. of washed sea sand. The residue was extracted with five 25 ml. portions of hot chloroform in which trimethylamine hydrochloride is soluble. The water-soluble fraction obtained by extracting the sand mixture with water weighed 256.4 mg.

The chloroform-soluble fraction (6.4 mg.) was treated with gold chloride solution. The analysis of the gold salt obtained on concentration agreed with that calculated for trimethylamine chloroaurate.

$\text{C}_3\text{H}_9\text{N} \cdot \text{HAuCl}_4$ . Calculated, Au 49.41; found, Au 48.91

*Identification of Choline*—The water-soluble residue was treated with gold chloride solution and the chloroaurate which formed was purified by several crystallizations (m.p.  $239\text{--}241^\circ$  with decomposition). A mixture with choline chloroaurate (m.p.  $243\text{--}244^\circ$  with decomposition) melted at  $241\text{--}243^\circ$  with decomposition. The analysis for gold agreed with that for choline chloroaurate.

$\text{C}_5\text{H}_{14}\text{NO} \cdot \text{AuCl}_4$ . Calculated, Au 44.50; found, Au 44.34

The chloroaurate which remained after analysis was decomposed with 0.5 gm. of finely divided silver and the base converted to the picrate. Small needles (m.p.  $238\text{--}240^\circ$  with decomposition) were characteristic of choline picrate. A mixture with an authentic sample of choline picrate (m.p.  $243\text{--}246^\circ$  with decomposition) melted at  $238\text{--}242^\circ$  with decomposition.

*Isolation of Unidentified Base*—The alkali-soluble reineckate was converted to the chloride (234.5 mg.). A chloroaurate was prepared and purified by crystallization (m.p.  $191\text{--}194^\circ$  with decomposition). The gold content was somewhat less than that calculated for betaine chloroaurate.

$\text{C}_5\text{H}_{11}\text{NO}_2 \cdot \text{HAuCl}_4$ . Calculated, Au 43.14; found, Au 42.37

The mother liquors from the precipitation and recrystallization of the chloroaurate were treated with finely divided silver to convert any bases present to the chloride. A picrate was prepared which melted at  $156\text{--}158^\circ$ .

Crystallization from alcohol yielded fine needles (m.p. 154–158°); betaine picrate melts at 181–183°, and homarine picrate at 159–161°. A mixture with homarine picrate lowered the melting point to 135–137°. Found, N 15.30.

#### SUMMARY

1. It was demonstrated that large scale collections of marine plankton may be made by the use of supercentrifuges.
2. A collection of marine plankton rich in *Gonyaulax catenella* was made in this manner and the poison was obtained by extraction.
3. Concentration of this poison by chromatography on acid-washed norit A furnished a fraction with a toxicity of 1.65 mouse units per microgram.
4. Choline and trimethylamine have been identified as two of the bases present in marine plankton.
5. A third base has been isolated but not identified.

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# THE AMOUNT OF COPPER BOUND BY PROTEIN IN THE BIURET REACTION\*

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(Received for publication, May 29, 1948)

In the course of a previous study of the biuret reaction (1) it occurred to us that the spectrophotometric data could be used to measure the amount of copper bound by the protein in the formation of the complex ion in this reaction. Such measurements would be of interest in establishing the structure of the complex (2), and are also required in order to evaluate the extinction coefficient of the copper-protein complex. Although the desired information can be obtained from the spectrophotometric data alone, it was felt that a more direct measure of the copper concentrations would be desirable. We have, therefore, combined spectrophotometric and polarographic data in much the same way that Borsook and Thimann (3) combined spectrophotometric and electrode potential data in their study of the combination of copper with amino acids.

## *Methods and Materials*

*Proteins*—Human serum albumin (Fraction V) and human serum  $\gamma$ -globulin (Fraction II) were obtained from the Department of Physical Chemistry, Harvard Medical School.<sup>1</sup>

Bovine serum albumin (Fraction V) and bovine serum  $\gamma$ -globulin (Fraction II) were obtained from the Armour Laboratories, Chicago. Dialyzed stock solutions of the albumins were prepared in distilled water, and stock solutions of the globulins were prepared in, and dialyzed against, 1 per cent NaCl. Concentrations of the stock solutions were determined both by drying to constant weight at 105° and by nitrogen determinations.

*Solutions for Study*—The solutions were made with a fixed concentration in the case of each protein. Stock solutions of NaOH and ethylene glycol were added to give the desired final concentration. The ethylene glycol was purified by distillation at atmospheric pressure. The reducing substances appear to polymerize and remain in the residue. The copper con-

\* Presented before the Thirty-first annual meeting of the Federation of American Societies for Experimental Biology at Chicago, May, 1947.

<sup>1</sup> These materials were prepared under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

centration was varied by adding increasing amounts of a stock solution of  $\text{CuSO}_4$ . All concentrations were made to contain the indicated number of gm. of protein, copper,  $\text{NaOH}$ , or ethylene glycol per 100 ml. of solution. All solutions were allowed to stand for at least 1 hour before measurements were made.

*Spectrophotometric Measurements*—A Beckman, quartz, photoelectric spectrophotometer with 1.00 cm. Corex cells was employed.

*Polarographic Measurements*—A Sargent Heyrovský polarograph was employed. It was found that satisfactory current-voltage curves could be obtained for copper in the presence of protein and concentrated  $\text{NaOH}$ , but

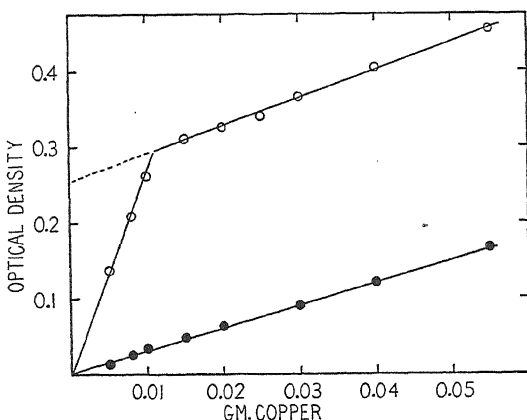


FIG. 1. Optical densities at 540  $m\mu$  of solutions of copper in 15 per cent  $\text{NaOH}$  as a function of the copper concentration in gm. per 100 ml. (●), and the same in the presence of human serum albumin at a concentration of 0.0882 gm. per 100 ml. (○).

not when ethylene glycol was also present. This might be related to the tendency of ethylene glycol to develop reducing substances. The copper concentrations were calculated from the ratio of the limiting current to that observed with a known copper concentration in the same concentration of  $\text{NaOH}$ , the limiting current being proportional to the copper concentration.

### Results

*Spectrophotometric Measurements*—Sample data for human albumin at 540  $m\mu$  are given in Fig. 1. As indicated in the previous publication (1), the optical density increases linearly with increasing copper concentration in the region of protein excess. When the copper is in excess, the optical density again increases in a linear fashion. It will be noted, however, that this second slope is somewhat greater than that for copper alone. Except



for intermediate portions of the curve, then, the optical densities of these solutions can be represented by two intersecting straight lines. In the

TABLE I

*Effect of Increasing Copper on Optical Density of Protein Solutions, with and without Ethylene Glycol*

Protein	Concentration	NaOH	Ethylene glycol	$k_1$	$k_2$	$k_3$	Intercept	
							Cu	Optical density
	gm. per 100 ml.	gm. per 100 ml.	gm. per 100 ml.				gm. per 100 ml.	
Bovine albumin	0.0767	5	0	25.2	0.218	3.28	0.0100	0.252
		15	0	25.2	0.218	3.28	0.0100	0.252
		5	2	22.5	0.187	3.48	0.0099	0.222
		15	2	23.5	0.193	3.56	0.0097	0.228
Bovine globulin ( $\gamma$ -)	0.0749	5	0	23.0	0.219	3.82	0.0113	0.237
		15	0	22.5	0.210	3.58	0.0111	0.250
		5	2	20.8	0.185	4.10	0.0111	0.230
		15	2	20.8	0.185	3.72	0.0108	0.225
Human albumin	0.0882	15	0	26.3	0.258	3.54	0.0115	0.303
		5	2	23.2	0.227	3.68	0.0117	0.272
Human globulin ( $\gamma$ -)	0.0840	15	0	22.5	0.228	3.48	0.0120	0.270
		5	2	20.1	0.202	3.64	0.0122	0.245

TABLE II

*Copper Bound by Bovine Albumin*

Bovine albumin, 0.0767 gm. per 100 ml.; NaOH, 15 gm. per 100 ml.

Total copper	Polarographic data		Spectrophotometric data		Protein per gm. copper	$\frac{(\text{Cu})_f (\text{Prot})_f^*}{(\text{Prot})_c}$
	Free copper	Combined copper	Combined protein	Free protein		
gm. per 100 ml.	gm. per 100 ml.	gm. per 100 ml.	gm. per 100 ml.	gm. per 100 ml.	gm.	
0.005	0.00056	0.00444	0.036	0.041	8.0	$6.4 \times 10^{-4}$
0.008	0.00076	0.0072	0.057	0.020	7.8	$2.7 \times 10^{-4}$
0.0100	0.00112	0.089	0.068	0.009	7.7	$1.4 \times 10^{-4}$
0.0150	0.0048	0.0102	0.073	0.004	7.2	$3 \times 10^{-4}$
0.0200	0.0093	0.0107	0.076	0.001	7.1	
0.0300	0.0188	0.0112	0.0767		6.8	
0.0500	0.0388	0.0112	0.0767		6.8	
0.0550	0.0438	0.0112	0.0767		6.8	

\* (Free copper  $\times$  free protein)/(combined protein).

interest of brevity, we are reporting only the constants which characterize these two linear functions at 540  $m\mu$ : region of protein excess, optical density =  $k_1(\text{Cu})$ ; region of copper excess, optical density =  $k_2 + k_3(\text{Cu})$ . The values of these constants are given in Table I.

It will be apparent that the curve for solutions which do not contain protein should be optical density =  $k_2 + k_3(\text{Cu})$ . In this case  $k_2 = 0$ , and in any of the solutions employed  $k_3 = 3.03 \pm 0.03$ . The fact that  $k_3$  is greater

TABLE III

*Copper Bound by Bovine Globulin*

Bovine globulin, 0.0749 gm. per 100 ml.; NaOH, 15 gm. per 100 ml.

Total copper	Polarographic data		Spectrophotometric data		Protein per gm. copper	$\frac{(\text{Cu})_f (\text{Prot})_f}{(\text{Prot})_0}$
	Free copper	Combined copper	Combined protein	Free protein		
gm. per 100 ml.	gm. per 100 ml.	gm. per 100 ml.	gm. per 100 ml.	gm. per 100 ml.	gm.	
0.005	0.00021	0.00479	0.0306	0.0444	6.4	$3.0 \times 10^{-4}$
0.008	0.00065	0.00735	0.0496	0.0204	6.8	$2.7 \times 10^{-4}$
0.010	0.00107	0.0089	0.062	0.013	7.0	$2.2 \times 10^{-4}$
0.015	0.0043	0.0107	0.071	0.004	6.7	$2.2 \times 10^{-4}$
0.020	0.0083	0.0117	0.073	0.002	6.3	
0.025	0.0135	0.0115	0.075		6.5	
0.030	0.0178	0.0122	0.075		6.2	
0.040	0.0264	0.0136	0.075		5.5	
0.050	0.0365	0.0135	0.075		5.6	

TABLE IV

*Copper Bound by Human Albumin*

Human albumin, 0.0882 gm. per 100 ml.; NaOH, 15 gm. per 100 ml.

Total copper	Polarographic data		Spectrophotometric data		Protein per gm. copper	$\frac{(\text{Cu})_f (\text{Prot})_f}{(\text{Prot})_0}$
	Free copper	Combined copper	Combined protein	Free protein		
gm. per 100 ml.	gm. per 100 ml.	gm. per 100 ml.	gm. per 100 ml.	gm. per 100 ml.	gm.	
0.0050	0.00049	0.00451	0.0400	0.048	8.9	$5.9 \times 10^{-4}$
0.0080	0.00049	0.00751	0.060	0.028	8.1	$2.3 \times 10^{-4}$
0.0100	0.00099	0.00901	0.072	0.016	8.0	$2.2 \times 10^{-4}$
0.0150	0.00346	0.0115	0.085	0.003	7.4	$1.1 \times 10^{-4}$
0.020	0.0079	0.0121	0.086	0.002	7.1	
0.025	0.0118	0.0132	0.087	0.001	6.6	
0.030	0.0168	0.0132	0.088		6.7	
0.040	0.0262	0.0138	0.088		6.4	
0.055	0.0400	0.0150	0.088		5.9	

than this in all of the solutions containing protein indicates that copper combines with protein in increasing amounts after the first complex is formed.

The values for copper concentration and optical density given under the heading "Intercept" in Table I are the values at the intersection of the

two linear components of each curve of optical density *versus* copper concentration, and represent the values for the completely saturated primary complex.

*Polarographic Data*—These data are given in Tables II to V. In Fig. 2, the bound copper, (total) — (free), is plotted as a function of the free copper. This latter method was employed to extrapolate the bound copper to zero copper concentration in order to eliminate the effect of the secondary binding of copper at higher copper concentration.

TABLE V  
*Copper Bound by Human Globulin*

Human globulin, 0.0840 gm. per 100 ml.; NaOH, 15 gm. per 100 ml.

Total copper	Polarographic data		Spectrophotometric data		Protein per gm. copper	$\frac{(\text{Cu})_f (\text{Prot})_f}{(\text{Prot})_c}$
	Free copper	Combined copper	Combined protein	Free protein		
<i>gm. per 100 ml.</i>	<i>gm. per 100 ml.</i>	<i>gm. per 100 ml.</i>	<i>gm. per 100 ml.</i>	<i>gm. per 100 ml.</i>	<i>gm.</i>	
0.005	0.00023	0.00477	0.0318	0.0522	6.7	$3.8 \times 10^{-4}$
0.008	0.00027	0.00773	0.055	0.029	7.1	$1.4 \times 10^{-4}$
0.010	0.00032	0.0097	0.068	0.016	7.0	$0.74 \times 10^{-4}$
0.015	0.00316	0.00118	0.079	0.005	6.7	$1.8 \times 10^{-4}$
0.020	0.0069	0.0131	0.082	0.002	6.3	
0.025	0.0135	0.0115	0.080	0.004	6.9	
0.030	0.0158	0.0142	0.084		5.9	
0.040	0.0244	0.0156	0.084		5.4	
0.055	0.0383	0.0167	0.084		5.0	

#### DISCUSSION

The results of these measurements may be considered most satisfactorily in terms of the hypothesis that the biuret reaction is characterized by the formation of a complex ion in which each atom of copper is bonded to 4 peptide nitrogens (2). The specific extinction, based on the copper concentration, would then be expected to be the same for all proteins. This value,  $k_1$  of Table I, is of the same order of magnitude in the cases examined and is not influenced by the NaOH concentration in the range studied. It does, however, depend upon the nature of the protein and is uniformly reduced about 10 per cent in the presence of 2 per cent ethylene glycol. The dependence upon the nature of the protein suggests that the proposed structure of the complex ion is either not entirely correct, or that the extinction is somewhat dependent upon the nature of the amino acids involved in the formation of the peptide bonds.

The amount of copper bound by the protein may be calculated in several ways. The intersection of the two curves obtained spectrophotometrically

and characterized by  $k_1$  and by  $k_2$  and  $k_3$  may be employed. Table I shows that this value is independent of the ethylene glycol and NaOH concentrations. It is, then, possible to calculate an average value for the quantity of each protein bound by 1 gm. of copper from the spectrophotometric data alone. A similar calculation may be made from the polarographic data alone. As indicated in Fig. 2, the bound copper may be plotted against the free copper, and the limiting straight line at high copper concentration

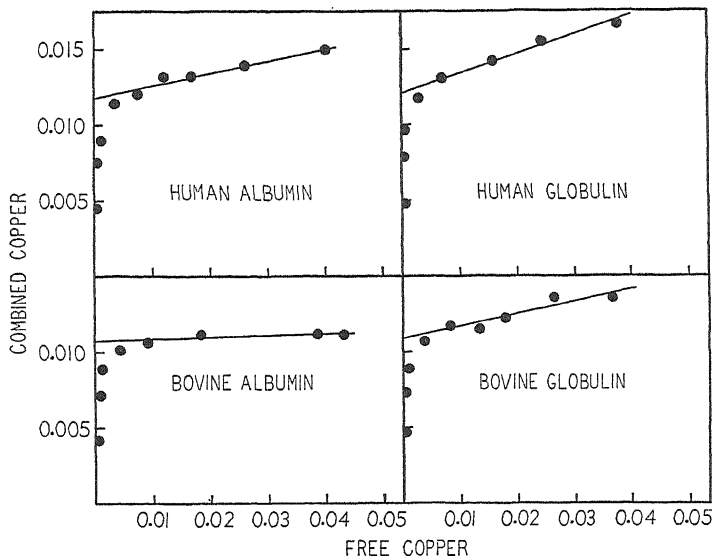


FIG. 2. The concentration of combined copper, calculated from the total copper concentration and the free copper determined polarographically as a function of the free copper concentration. Human serum albumin, 0.0882 gm. per 100 ml.; human  $\gamma$ -globulin, 0.0840 gm. per 100 ml.; bovine serum albumin, 0.0767 gm. per 100 ml.; bovine  $\gamma$ -globulin, 0.0749 gm. All solutions contain 15 gm. of NaOH per 100 ml. The copper concentration is in gm. per 100 ml.

extrapolated to the value for the bound copper at zero free copper concentration. Finally, the two sets of data may be combined, by using the difference between total and free copper as determined polarographically to calculate the combined copper. The combined protein at the same total copper concentration can be calculated by multiplying the total protein concentration by the ratio between the observed optical density and the optical density calculated from  $k_2$  and  $k_3$ . At each total copper concentration, then, the gm. of protein per gm. of copper in the complex can be calculated. These calculations are given in Tables II to V.

The average residue weights obtained by dividing the amount of protein

per gm. atom of copper by 4 are given in Table VI. They are compared with the average residue weights calculated from the amino acid analysis by Brand (4). The agreement appears to be good enough to justify the conclusion that 4 peptide bond nitrogens are bonded to each copper atom in the complex ion of the biuret reaction. Differences between the extinction coefficients for different proteins can, then, be most probably attributed to the influence of the nature of the amino acids making up the peptide bonds. Some preliminary studies of gelatin support this view.

The second point of interest relates to the evidence for a secondary copper binding, probably by groups other than peptide nitrogens. As judged by the values of  $k_3$  in Table I, this second effect is least with bovine albumin, and increases in the order of human globulin, human albumin, and bovine

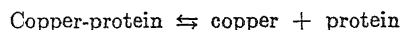
TABLE VI  
*Average Residue Weights\**

Protein	Spectropho- tometric	Polaro- graphic	Combined	Analytical, Brand (4)
Bovine albumin.....	123	111	121	119
“ $\gamma$ -globulin.....	108	113	106	
Human albumin.....	121	117	132	119
“ $\gamma$ -globulin.....	110	111	111	114

\* Protein per gm. atom of copper divided by 4.

globulin. The polarographic data in 15 per cent NaOH give rates of increase of copper bound per gm. of protein for each 0.1 gm. increase in copper concentration of essentially 0, 0.15, 0.09, and 0.12 gm. for bovine albumin, human globulin, human albumin, and bovine globulin when copper is in excess. The data do not justify any attempt to interpret differences between proteins, but they do provide an explanation for the failure of the relation between protein concentration and extinction to follow Beer's law under all circumstances (1). Although the biuret complex may be readily saturated with copper, and its absorption presumably would obey Beer's law under the conditions generally employed, the amount of the second type of complex formed will depend upon the relative proportions of "free" copper and protein in the solution and will be relatively less at higher protein concentrations when more copper is used up in forming the biuret complex.

The order of magnitude of the dissociation constant of the biuret complex may also be obtained from these data. Although it represents an oversimplification, the dissociation may be written



If we express the concentrations of both the complex and the protein in gm. per 100 ml., the concentration units will cancel, and (Cu) may be expressed in moles per liter. The dissociation constants calculated in this way are given in the last columns of Tables II to V.

Several general points bearing on the use of the biuret reaction in protein determinations may also be mentioned. It appears that the quantitative value of the extinction coefficient is not simply a function of the number of peptide bonds available in different proteins. Except for simplicity, the biuret method offers no particular advantage over other colorimetric methods for the determination of protein concentrations, since standards must be determined for each protein used. The magnitude of this difference is indicated by differences between values of  $k_1$ , since  $k_1$  represents the specific extinction related to copper concentration and hence to the peptide bond concentration. Human albumin and globulin, for example, differ by 15 per cent. It will be noted that the extinction coefficients for human albumin and globulin in 5 per cent NaOH and 2 per cent ethylene glycol are considerably higher than the values previously reported for mixed plasma proteins (1). These higher values have also been obtained with fresh human plasma. Although we have not reinvestigated the point, we feel more inclined to attribute the differences to some change in stored, liquid plasma used in the earlier study than to an analytical error. Finally, there does not seem to be any reason to prefer the use of ethylene glycol and lower NaOH concentrations to the use of higher concentrations of NaOH alone, as suggested by Kingsley (5), in biuret reagents for quantitative protein determinations.

#### SUMMARY

1. The extinction coefficients of solutions of human and bovine albumin and  $\gamma$ -globulin have been measured in alkaline solutions as a function of increasing copper concentration.

2. The free copper concentration has also been determined polarographically.

3. Average residue weights, calculated from these data on the assumption that each atom of copper binds 4 peptide nitrogens, are 118 for bovine albumin, 109 for bovine  $\gamma$ -globulin, 123 for human albumin, and 111 for human  $\gamma$ -globulin.

4. It is pointed out that the data demonstrate additional binding of copper which is less strong and probably involves different groups from those concerned in the biuret reaction.

5. The relation of these findings to the use of the biuret method in the quantitative determination of proteins is discussed.

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# THE EFFECT OF CERTAIN SULFONAMIDES UPON LACTOBACILLUS ARABINOSUS IN A NICOTINIC ACID-RESTRICTED MEDIUM\*

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(Received for publication, June 5, 1948)

The extensive use of microbiological procedures for the determination of vitamins is well known. Although most of these methods have rather general applicability, it is possible that the conditions of a particular experiment might require alteration of the medium in order to insure its adequacy for the samples being analyzed. Such a situation might well arise in connection with the *Lactobacillus arabinosus* assay for nicotinic acid in the presence of sulfonamides (1). The importance of this fact is appreciated when one considers that the administration of sulfonamides, which cause a decrease in the synthetic activity of the intestinal flora, has become a commonly used and valuable means of studying the requirements for certain vitamins (2). Studies of this type often include fecal vitamin analyses (3-7) for which, in many instances, microbiological procedures are employed. Since the sulfonamide employed in such an investigation is generally chosen for its slow absorption and high bacteriostatic activity (7), its presence in the feces is assured and its possible effect upon the assay organism must be considered. According to the level of sulfathalidine administered to cows in a recent study at this Experiment Station, as much as 5 mg. of this compound might be present per gm. of feces, which would result in at least 0.5 mg. per sample taken for microbiological assay. It is possible that considerably higher concentrations might occur in other similar experiments. A study of this problem in connection with the *Lactobacillus arabinosus* assay for nicotinic acid has shown that within certain limits the sulfonamide effect varies with the amount of nicotinic acid present and, as was previously demonstrated by Teply *et al.* (1), can be prevented by the inclusion of additional *p*-aminobenzoic acid in the basal medium.

## EXPERIMENTAL

This study was primarily concerned with a determination of the extent to which the presence of sulfonamides in assay samples might affect the

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accuracy of microbiological determinations of nicotinic acid. The commonly used assay procedure of Snell and Wright (8) as modified by Krehl, Strong, and Elvehjem (9) was followed with the exception that final readings were made by determination of pH (10) rather than by titration. In this method the final results of an assay are obtained by reference to a standard curve which represents the acid production by *Lactobacillus arabinosus* when incubated at 35° for 72 hours in the presence of graded amounts of nicotinic acid. Since it was believed that the intensity of any sulfonamide effect encountered might vary with the nicotinic acid level, determinations were made in the presence of varying concentrations of this vitamin. The concentrations employed were 0.0, 0.1, 0.2, 0.3, and 0.4  $\gamma$  per assay tube and were sufficient to cover the range used in preparing the standard assay curve. With each concentration of sulfonamide the pH of the assay tube containing no nicotinic acid was found not to differ

TABLE I

*Acid Production by Lactobacillus arabinosus in Presence of Sulfanilamide*  
The recorded values are pH readings.

Nicotinic acid	Standard curve	Sulfanilamide				
		2.0 mg.	4.0 mg.	6.0 mg.	8.0 mg.	10.0 mg.
$\gamma$						
0.0	5.10	5.07	5.11	5.11	5.11	5.11
0.1	4.53	4.50	4.49	4.51	4.52	4.50
0.2	4.31	4.28	4.26	4.30	4.28	4.26
0.3	4.16	4.14	4.12	4.18	4.16	4.28
0.4	4.06	4.06	4.11	4.27	4.29	4.32

significantly from the corresponding sample of the standard curve. It was therefore concluded that the sulfonamides themselves did not affect the pH readings. The sulfonamides studied were sulfanilamide, sulfapyridine, sulfathalidine, and sulfadiazine, and their concentrations likewise were varied in carrying out the assays.

#### RESULTS AND DISCUSSION

The results obtained when determinations were carried out in the presence of varied sulfonamide concentrations are presented in Tables I to IV. These data show that sulfonamides, if present in sufficient concentration, retard acid production by *Lactobacillus arabinosus*. The sulfonamide effect was greatest, and manifested itself at the lowest concentration, in those tubes which contained the greatest amount of nicotinic acid. In all cases there was a direct relationship between this effect and the nicotinic acid concentration. This direct proportionality is readily

explained when one considers that the concentration of *p*-aminobenzoic acid (PABA) was constant throughout all determinations. It has been

TABLE II

*Acid Production by Lactobacillus arabinosus in Presence of Sulfapyridine*  
The recorded values are pH readings.

Nicotinic acid	Standard curve	Sulfapyridine				
		2.0 mg.	4.0 mg.	6.0 mg.	8.0 mg.	10.0 mg.
$\gamma$						
0.0	5.11	5.08	5.14	5.14	5.12	5.13
0.1	4.58	4.53	4.56	4.54	4.53	4.71
0.2	4.34	4.28	4.34	4.32	4.52	4.78
0.3	4.24	4.21	4.24	4.22	4.44	4.83
0.4	4.05	4.09	4.31	4.38	4.83	4.91

TABLE III

*Acid Production by Lactobacillus arabinosus in Presence of Sulfathalidine*  
The recorded values are pH readings.

Nicotinic acid	Standard curve	Sulfathalidine				
		2.0 mg.	4.0 mg.	6.0 mg.	8.0 mg.	10.0 mg.
$\gamma$						
0.0	5.40	5.43	5.44	5.40	5.37	5.41
0.1	4.65	4.63	4.67	4.64	4.66	4.64
0.2	4.36	4.36	4.39	4.36	4.60	4.58
0.3	4.21	4.22	4.29	4.34	4.37	4.41
0.4	4.09	4.12	4.40	4.40	4.44	4.47

TABLE IV

*Acid Production by Lactobacillus arabinosus in Presence of Sulfadiazine*  
The recorded values are pH readings.

Nicotinic acid	Standard curve	Sulfadiazine				
		2.0 mg.	4.0 mg.	6.0 mg.	8.0 mg.	10.0 mg.
$\gamma$						
0.0	5.42	5.42	5.37	5.44	5.38	5.42
0.1	4.71	4.73	4.71	4.76	4.68	4.70
0.2	4.39	4.41	4.34	4.37	4.37	4.52
0.3	4.26	4.26	4.26	4.22	4.36	4.40
0.4	4.12	4.11	4.08	4.32	4.30	4.39

shown (11) that PABA is necessary for optimal acid production by *Lactobacillus arabinosus*. Furthermore, it has been established that the anti-

bacterial activity of sulfanilamide, and other related compounds, is due to an inhibition of PABA utilization (12). It is apparent, therefore, that a definite amount of PABA would be more nearly completely used up in those samples containing high concentrations of nicotinic acid, in which, consequently, growth of the organism was profuse. Conversely, in those samples in which growth of the organism was restricted by a decreased nicotinic acid concentration, the PABA present was less rapidly used up and, therefore, more sulfonamide was required to inhibit its function. It appears entirely logical to believe that this situation is not peculiar to nicotinic acid, but rather that it would be effected by any other factor, the restriction of which inhibits growth of the organism and the production of acid.

TABLE V

*Effect of PABA on Suppression of Acid Production Caused by Sulfathalidine*  
The recorded values are pH readings.

Nicotinic acid	Standard curve	10 mg. sulfathalidine per tube		
		No PABA	Added PABA	
			10 $\gamma$	100 $\gamma$
$\gamma$				
0.0	5.52	5.48	5.48	5.52
0.1	4.58	4.58	4.61	4.60
0.2	4.29	4.40	4.32	4.31
0.3	4.15	4.42	4.17	4.17
0.4	4.08	4.24	4.11	4.10

The degree of inhibition of acid production is not the same for all compounds studied. This is in agreement with the established fact that sulfonamides differ in their antibacterial activity. Thus it is seen that the least effective compound is sulfanilamide. Under the conditions of this experiment both sulfathalidine and sulfapyridine were considerably more effective in inhibiting acid production, while the effect of sulfadiazine was intermediate. These differences are most marked under conditions of nicotinic acid restriction, while the amount required to inhibit acid production in the presence of the highest concentration of this vitamin is approximately the same in all cases.

The data of Table V are in agreement with the previously established (1) fact that the sulfonamide effect can be overcome by increasing the concentration of PABA in the medium. At the three highest nicotinic acid levels the pH readings definitely indicate decreased acid production in the presence of 10 mg. of sulfathalidine. Addition of as little as 10  $\gamma$

of PABA, however, results in pH readings which agree favorably with those of the standard curve.

Because of difficulty in effecting solution in some cases, particularly when the higher concentrations were used, it is not possible, from the present data, to establish absolute mathematical relationships between the various sulfonamides. However, the results reported herein represent conditions as they would occur if such compounds were present in an assay sample and indicate that the determination would be unaffected by the presence of as much as 2 mg. of the sulfonamide. In the presence of greater sulfonamide concentrations, the addition of an appropriate quantity of PABA to the medium will result in satisfactory assays.

#### SUMMARY

In the microbiological assay for nicotinic acid with *Lactobacillus arabinosus*, results are not affected by the presence of as much as 2 mg. of sulfanilamide, sulfapyridine, sulfathalidine, or sulfadiazine if the medium contains 0.2  $\gamma$  of PABA per ml. Higher concentrations of the sulfonamides inhibit acid production by the organism, and this inhibition, within certain limits, varies with the level of nicotinic acid. This sulfonamide effect can be prevented by increasing the amount of PABA in the basal medium.

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# ESSENTIAL AMINO ACID COMPOSITION OF SOY BEAN MEALS PREPARED FROM TWENTY STRAINS OF SOY BEANS\*

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(Received for publication, June 11, 1948)

The specific varieties of the most important food plants which are grown today were obtained through careful selection and breeding. Breeding experiments have demonstrated that the protein content of seed crops is subject to variation, and therefore suggest the possibility that the nutritional quality of seed proteins might be influenced by breeding. A change in distribution of the essential amino acids, as a result either of the production of different proteins or of variation in the relative proportions of the individual proteins in the seeds of different varieties, could definitely alter the nutritive value of the total seed protein.

Investigations with different strains of soy beans have demonstrated considerable variation in the total protein content of the seed. Cartter and Hopper (1) examined ten varieties and found protein values ranging from 36.6 to 53.2 per cent. Less extreme variations in the protein content within a given strain as a result of environmental factors have also been observed (1-6). Since soy bean culture is limited to a small group of select varieties which are grown principally in the corn belt states and are commonly pooled in the processing of the seed for oil and meal production (7), variations which result from environmental conditions probably have little effect on the protein content and composition of commercial soy bean meals. However, in view of the wide variation noted in protein content among different strains of soy beans one might expect significant variations to occur in amino acid composition of different strains. Csonka and Jones (8) reported variations in the cystine, tryptophan, and tyrosine content of soy bean seeds of six varieties in 1934 and suggested that "varieties should be selected for planting which produce more and better quality protein from the nutritional standpoint."

Doty *et al.* (9) have recently reported analyses of corn single crosses for tryptophan, tyrosine, cystine, arginine, and histidine and have concluded

\* This investigation was supported by a grant from The Procter and Gamble Company.

that the amount of these amino acids in the corn protein is related to the genetic constitution of the plant.

Microbiological methods of assay for the essential amino acids can now be conveniently applied to this problem. A study was therefore made of the essential amino acid content of soy bean meals prepared from twenty different strains of soy beans to determine whether there may be outstanding differences in composition of nutritional importance.

#### *Materials and Methods*

*Preparation of Soy Bean Meals*—The soy bean meals analyzed in this investigation were prepared by The Procter and Gamble Company from soy beans obtained through the cooperation of the United States Regional Soybean Laboratory at Urbana. Carefully composited samples were prepared so that location and soil differences would cancel as nearly as possible. The group of twenty strains includes the most promising varieties that are now being grown and others that are being considered for release.

The meals were all prepared by solvent extraction under comparable conditions and were supplied as raw and toasted flakes. Toasting was accomplished by heating the raw flakes in a steam-jacketed container at atmospheric pressure for 30 minutes. The temperature ranged from 21–88° during the first 12 minutes, 6 minutes later reached 104°, and was held between 104–110° for 12 additional minutes. The original moisture content of the toasted flakes was 20 per cent; the final moisture content of the toasted flakes was approximately 11 per cent.

*Methods of Analysis*—Microbiological methods of amino acid assay were employed. Valine, leucine, isoleucine, and glutamic acid were determined with *Lactobacillus arabinosus* 17-5 (10, 11). *Leuconostoc mesenteroides* P-60 was used to determine methionine (12), arginine, lysine, and phenylalanine.<sup>1</sup> *Streptococcus faecalis* R was used to determine threonine, histidine (13), and tryptophan (14).

Hydrolysates for tryptophan analyses were prepared with NaOH and cysteine as previously described (14). Acid hydrolysates used for the determination of the other amino acids were prepared by suspending 1 gm. of sample in 100 ml. of 6 N HCl and gently refluxing for 24 hours. After removing the excess acid by vacuum distillation, the hydrolysates were diluted to 100 ml., filtered, and refrigerated. Aliquots were neutralized for analysis.

#### RESULTS AND DISCUSSION

Microbiological methods of amino acid analysis are particularly useful to the nutritionist, because they can be applied directly to the analysis of

<sup>1</sup> Kuiken, K. A., and Lyman, C. M., unpublished methods.



complex foodstuffs. Since it is not necessary to isolate specific protein fractions, it is possible to evaluate the total amino acid content of a foodstuff which contains a mixture of individual proteins in different proportions. Difficulty is experienced, however, in arriving at a satisfactory method of expressing analytical data for such products. If one cites the amino acid content of the foodstuff, he must also provide other data on gross composition to facilitate comparison of different samples. This can best be done by calculating all data to some common nitrogen basis. The practice of calculating to 16 per cent nitrogen has been widely used even though it is now recognized that the nitrogen content of most proteins is not 16 per cent. Glycinin, the principal protein of soy beans, contains 17 per cent nitrogen (15). Smiley and Smith (16) found from 16.26 to 16.90 per cent nitrogen in "soybean protein." Nevertheless, the exact nitrogen content of the total soy bean seed protein is not known and there seems little value in adopting a new factor for calculating the data. An alternative method of expressing the data is to calculate the ratio of amino acid nitrogen to total nitrogen. This avoids an empirical assumption and permits comparison of different samples. However, this method does not in itself provide a measure of the amino acid content of the sample and is not as useful to the nutritionist as data calculated on the crude protein basis. In view of these considerations, the authors have expressed all amino acid data in this paper in terms of 16 per cent nitrogen.

The soy bean meals were analyzed for the ten essential amino acids and glutamic acid. The content of these amino acids in raw soy bean meals prepared from twenty different strains of soy beans is given in Table I. No outstanding differences in individual amino acid content were observed. The greatest variation occurred in methionine content, of which the highest value was 19 per cent above the lowest. With most of the other amino acids, the variations were considerably smaller. The data suggest a great degree of uniformity in amino acid composition of the soy bean seed, rather than extensive variation as suggested by the work of Csonka and Jones (8).

Portions of a second lot of soy bean meals were toasted in order to inactivate the trypsin inhibitor shown to be present in raw soy beans by Bowman (17) and Ham and Sandstedt (18). The essential amino acid content of the toasted samples was compared with identical raw controls. As is shown in Table II, there was practically no change in amino acid content as a result of toasting, with the possible exception of lysine. The average data indicate that about 5 per cent of the lysine in the soy bean meals may have been destroyed by the heat treatment. The observations with lysine were, therefore, extended to include analyses of raw and toasted samples of soy bean meals representing twenty-one strains of soy beans. The additional data (Table III) indicate a similar small loss of lysine which

TABLE I  
*Amino Acid Content of Raw, Solvent-Extracted Soy Bean Meals Prepared from Twenty Strains of Soy Beans*  
 The values are expressed as the percentage of the amino acid in the crude protein (equivalent to calculating to 16 per cent N).

Soy bean strain	Crude protein (N $\times$ 6.25)	Arginine	Glutamic acid	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenyl- alanine	Threonine	Tryptophan	Valine
Acadian....	48.89	7.75	18.1	2.29	5.34	7.75	6.52	1.43	4.95	3.95	1.51	5.44
Arksoy....	50.92	7.56	18.9	2.30	5.30	7.86	6.54	1.39	5.11	3.87	1.45	5.30
A3-176....	48.84	7.68	18.6	2.25	5.34	7.90	6.70	1.43	5.02	3.93	1.56	5.43
A3K-884....	47.24	8.09	18.5	2.26	5.42	7.85	6.88	1.40	4.87	4.06	1.50	5.31
A4-107-12....	47.95	8.01	18.2	2.23	5.22	7.93	6.53	1.34	4.84	3.84	1.48	5.28
Chief.....	48.23	7.82	18.4	2.30	5.20	7.86	6.59	1.48	5.04	3.96	1.56	5.41
C. N. S.....	50.84	7.87	18.4	2.36	5.19	7.59	6.00	1.31	5.11	3.72	1.55	5.35
C-463.....	47.24	7.54	18.0	2.37	5.39	7.96	6.90	1.45	5.07	3.96	1.61	5.40
Earlyana....	48.81	7.72	18.7	2.29	5.31	7.95	6.74	1.37	5.20	3.98	1.56	5.31
Gibson.....	48.19	7.49	18.6	2.30	5.35	8.13	6.91	1.41	5.08	3.82	1.54	5.31
H-5.....	49.42	7.22	17.9	2.16	5.24	7.97	6.51	1.38	5.04	3.58	1.54	5.22
Lincoln....	48.86	7.53	18.4	2.29	5.32	8.08	6.67	1.40	5.22	3.91	1.64	5.42
Lincoln No. 3.....	46.38	7.72	19.2	2.33	5.43	8.45	6.73	1.53	5.17	4.03	1.60	5.48
Mamloxi....	49.49	7.96	18.3	2.40	5.53	7.94	7.07	1.50	5.23	3.86	1.44	5.34
N44-92....	48.96	7.60	18.5	2.49	5.29	8.17	6.70	1.35	5.17	4.04	1.49	5.47
N44-774....	46.38	7.85	17.9	2.37	5.28	8.04	6.73	1.35	5.13	3.99	1.57	5.17
Ogden.....	47.81	7.49	18.5	2.34	5.48	8.16	6.76	1.28	5.31	3.83	1.42	5.31
Richland....	46.38	8.30	18.4	2.35	5.18	7.98	6.47	1.37	4.80	3.84	1.57	5.20
Roanoke....	47.40	7.64	19.0	2.47	5.15	8.02	6.48	1.41	5.23	3.88	1.48	5.23
S-100.....	49.98	7.56	18.6	2.52	5.32	7.98	6.54	1.42	5.12	3.76	1.46	5.32
Average*.		7.72 $\pm 0.055$	18.4 $\pm 0.07$	2.33 $\pm 0.020$	5.31 $\pm 0.023$	7.98 $\pm 0.039$	6.65 $\pm 0.050$	1.40 $\pm 0.013$	5.08 $\pm 0.031$	3.90 $\pm 0.026$	1.53 $\pm 0.013$	5.34 $\pm 0.020$

\*  $\pm$  standard error.

TABLE II  
*Effect of Toasting on Amino Acid Content of Solvent-Extracted Soy Bean Meals*  
 The values are expressed as the percentage of amino acid in crude protein.

Amino acid	A3K-884		C-463		Mamloxi		Memphis blank		Richland		Average	
	Raw	Toasted	Raw	Toasted	Raw	Toasted	Raw	Toasted	Raw	Toasted	Raw	Toasted
Arginine.....	7.92	7.81	7.56	7.63	7.70	7.86	8.03	8.17	8.10	8.09	7.86	7.91
Glutamic acid.....	18.2	18.3	18.2	18.2	18.5	18.6	18.8	18.4	18.2	18.1	18.4	18.3
Histidine.....	2.37	2.43	2.48	2.51	2.50	2.48	2.40	2.26	2.47	2.37	2.44	2.41
Isoleucine.....	5.42	5.38	5.39	5.42	5.48	5.34	5.28	5.21	5.18	5.23	5.35	5.32
Leucine.....	7.64	7.77	7.73	7.71	7.80	7.77	7.66	7.64	7.57	7.47	7.68	7.67
Lysine.....	6.50	6.23	6.90	6.38	7.07	6.49	6.56	6.40	6.35	6.32	6.68	6.36
Methionine.....	1.38	1.39	1.44	1.48	1.45	1.39	1.43	1.40	1.38	1.40	1.42	1.41
Phenylalanine.....	4.78	4.80	4.92	5.00	5.30	5.32	4.80	4.86	4.85	4.75	4.93	4.95
Threonine.....	4.06	4.07	3.96	3.89	3.95	4.17	3.95	3.80	3.81	3.95	3.95	3.98
Tryptophan.....	1.50	1.50	1.61	1.65	1.45	1.49	1.55	1.56	1.43	1.48	1.51	1.55
Valine.....	5.23	5.24	5.22	5.25	5.32	5.28	5.32	5.38	5.16	5.12	5.25	5.25

would not be expected to affect significantly the nutritive value of the toasted material. However, since the difference in the mean values for the paired raw and toasted samples exceeded the standard error of the difference between the two means by a factor of 5, the result is highly significant statistically (19). The work of other authors indicates that the lysine in soy bean meals can be extensively destroyed by overheating. Riesen *et al.* (20) found that about 50 per cent of the lysine in a soy bean meal sample was destroyed by autoclaving for 4 hours at 15 pounds pressure. Evans and McGinnis (21) observed 30 per cent destruction of lysine in a meal that was autoclaved for 1 hour.

TABLE III

*Effect of Toasting on Lysine Content of Solvent-Extracted Soy Bean Meals*

The values are expressed as the percentage of lysine in crude protein.

Soy bean strain	Raw	Toasted	Soy bean strain	Raw	Toasted
Acadian	6.52	6.21	H-5	6.51	6.11
Arksoy	6.54	6.43	Lincoln	6.67	6.21
A3-176	6.70	6.55	Lincoln No. 3	6.73	6.06
A4-107-12	6.53	6.38	N44-92	6.70	6.18
Chief	6.59	6.89	N44-774	6.73	6.04
C. N. S.	6.00	6.07	Ogden	6.76	6.22
Earlyana	6.74	6.23	Roanoke	6.48	6.26
Gibson	6.91	6.42	S-100	6.54	6.21
Average*.....				6.60	6.28

\* Composite averages  $\pm$  standard errors for lysine in the total of twenty-one samples represented in Tables II and III are  $6.62 \pm 0.048$  and  $6.30 \pm 0.043$  for the raw and toasted meals respectively.

Recent values from the literature for the essential amino acid content of soy bean meal are given in Table IV. Only values obtained by microbiological methods are recorded. In general, there is very good agreement with the values obtained in this investigation. The most striking discrepancy is in the methionine value reported by Stokes (23). The application of Stokes' method in the authors' hands has in general given low values for methionine. Lysine values reported by Stokes (23), Baumgarten (22), and Schweigert (24) are lower than those by Riesen (20) and the authors. Perhaps this is an indication of slight overheating in the samples, which gave low values. The authors' data and those of Riesen refer to raw preparations. It is conceivable that lysine assays might be used to detect overheating of soy bean meal. The high values for tryptophan reported by the present authors were obtained by an improved method of hydrolysis

(Kuiken *et al.* (14)). It is evident that soy bean meals sampled independently tend to have identical amino acid composition.

The relatively small variations in the content of the individual amino acids in the protein of the different varieties of soy beans is paralleled by the authors' (26) findings with respect to cottonseed meal. Eight samples of cottonseed meal collected from different parts of the country were found to have practically the same amino acid composition when the data were calculated to 16 per cent nitrogen.

TABLE IV

*Comparison of Present Data with Recent Literature Values for Essential Amino Acid Content of Soy Bean Meal*

All data were obtained by microbiological methods and are expressed here as the percentage of amino acid in the crude protein.

Amino acid	Present paper	Riesen (20)	Baumgarten (22)	Stokes (23)	Schweigert (24, 25)
Arginine.....	7.7	7.4	5.3	7.1	7.3
Histidine.....	2.3	2.8	3.0	2.3	2.2
Isoleucine.....	5.3	5.1	6.2	4.5	4.8
Leucine.....	7.9	7.6	7.9	7.4	7.1
Lysine.....	6.6	6.6	5.3	5.4	5.7
Methionine.....	1.4	1.4	1.6	0.8	
Phenylalanine.....	5.1	5.1	4.9	5.3	4.5
Threonine.....	3.9	3.8	3.7	3.9	3.3
Tryptophan.....	1.5	1.2	1.1	1.2	
Valine.....	5.3	5.4	5.3	4.6	5.2

## SUMMARY

Soy bean meals prepared from twenty strains of soy beans were analyzed for the essential amino acids and glutamic acid by microbiological methods. The amino acid distribution in the crude protein of the different samples was in general quite uniform. The greatest variation occurred in the case of methionine, in which the highest value was 19 per cent above the lowest.

Toasting the meals as practiced by the industry in order to inactivate the proteolytic inhibitor of raw soy beans did not result in the loss of any amino acids except lysine. The loss of lysine indicated by the data was too small to be of nutritional significance.

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# MICROGRAM ANALYSIS: FURTHER STUDIES OF DETERMINATION OF GLUCOSE AND ITS APPLICATION TO THE DETERMINATION OF SUCROSE\*

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(Received for publication, June 18, 1948)

Commonly, microanalysis of plant sugars is conducted on a mg. scale (1); on the microgram level, a general method appears to be lacking. Some colorimetric methods suitable for determination of microgram quantities of sugars are available, but for a combination of high sensitivity and good accuracy these are not very satisfactory. Anthrone, for example, has been used recently to determine sucrose in amounts as low as  $2\gamma$  (2), but there is no indication by the author as to the degree of accuracy possible. A summary of the various color reactions for different sugars by Umbreit (3) indicates a lower limit in all cases of  $10\gamma$  with an accuracy of  $\pm 4$  per cent. Our aim has been to make possible the analysis of sugars in amounts as low as  $1\gamma$ , with a maximum variation for single determinations of  $\pm 2$  per cent.

Since non-reducing sugars are easily hydrolyzed to reducing ones, the problem is largely one of determining reducing sugar. This has already been solved by Heck, Brown, and Kirk (4) titrimetrically by the use of ferricyanide and ceric sulfate, a method inherently more accurate than colorimetric procedures. In the original work, however, a number of small factors were overlooked which nevertheless strongly limited the accuracy of determinations, especially at the lower limits of the method. Our present report deals, therefore, with these accessory points of technique and further describes the adaptation of the method to sucrose analysis. In an accompanying publication methods of extraction and separation of sugars from plant tissues with weights of the order of 1 mg. are discussed.

## EXPERIMENTAL

### *Glucose*

*Magnitude of Blank Titrers*—On the mg. scale the reducing power of the blank (potassium ferricyanide plus sodium carbonate) is relatively small and its exact value, therefore, is of minor importance. In the microgram

\* Aided by grants from the United States Public Health Service, the Research Board of the University of California, and the Royal Society of Canada.

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range this is not the case; in fact, in the original studies it was found that often the blank titer exceeded that equivalent to 1  $\gamma$  of glucose. This disadvantage can be overcome by addition of exact amounts of oxidizing agent. The procedure is undesirable, however, since, apart from adding tediousness to the method, the total volume of the solution is increased due to the necessary rinsing of pipettes. The alternative, reduction in magnitude of blank, was therefore sought, and the factors affecting it are summarized below.

TABLE I

*Effect of Ferricyanide Concentration on Blank Titer*

The volumes indicated were taken from a 1.5 per cent solution, equal volumes of 4 per cent sodium carbonate added, and the mixture made up to about 200  $\lambda$ . These were titrated with 0.0089 N ceric sulfate.

Volume of ferricyanide	Ceric sulfate
$\lambda$	$\lambda$
40	2.85
25	1.89
20	1.12
10	0.60

TABLE II

*Effect of Sodium Carbonate Concentration on Blank Titer of 40  $\lambda$  of 0.8 Per Cent Potassium Ferricyanide*

Concentration of added carbonate	Ceric sulfate
<i>per cent</i>	$\lambda$
2	0.84
14	1.65
30	2.70
14 + 40 $\lambda$ 5% HCl	1.78

*Concentration of Ferricyanide*—The relation between reducing power of blank and amount of ferricyanide present is roughly proportional. The results are shown in Table I. In our procedure, therefore, we replaced the original 1.5 per cent ferricyanide solution with one which was 0.8 per cent, a concentration ample for quantities as high as 12  $\gamma$  of glucose. Furthermore, when the range of the unknown sugar was 1 to 6  $\gamma$ , it was found expedient to add half as much oxidizing agent.

*Sodium Carbonate*—Although this reagent is necessary to maintain alkalinity of the medium, the concentration previously used (14 per cent) was excessive. Sodium carbonate alone has no reducing power, but from Table II it can be seen that an increase in concentration of carbonate in



the presence of ferricyanide is paralleled by an increase in blank titer. It may also be noted that partial removal of carbonate by adding HCl to the blank before heating on the steam bath again decreases the blank value. Thus, since the presence of 2 per cent carbonate in the standard ferricyanide solution provided sufficient alkalinity, the use of 14 per cent sodium carbonate was discontinued.

*Age of Ferricyanide Solution*—In the earlier publication (4) it was pointed out that aging of ferricyanide had no effect on results. This, however, would appear to be true only with respect to the amount of ferrocyanide produced in oxidation of the sugars. The blank titer itself increases with age of reagent. We, therefore, adopted Hassid's procedure (1) of storing the reagent in the cold and usually made up a new stock solution every week.

*Additional Points of Technique.* (a) *Drying of Pipettes*—The use of an unfiltered air-stream for drying the pipettes is to be avoided. The practice, in fact, was found to be a major source of error in the original work when frequently abnormally large blank values were obtained. It is not clear what, in the air, increases the reducing power of the solution, but the effect is eliminated either by rinsing the pipettes several times before use, or drying with filtered air. In standardizing our procedure, we dried only those pipettes used for delivering definite volumes of the sugar solutions; for all reagents the pipettes were first rinsed with distilled water, and then with the reagent itself.

(b) *Effect of Volume*—In the original publication it was considered necessary to make all samples up to a standard volume (200  $\lambda$ ).<sup>1</sup> Our results indicate that the practice may be omitted. This is shown in Table III along with the effects of rinsing and air-drying the pipettes.

*Reagents, Revised Method*—(1) *Potassium ferricyanide solution*, 0.8 per cent, containing 2 per cent sodium carbonate. (2) *Standard ceric sulfate*, about 0.01 N (for preparation see (4)). (3) *Sulfuric acid*, 10 per cent by volume.

*Procedure*—The sample containing 1 to 12  $\gamma$  of glucose is delivered to a micro test-tube, 3 mm. inside diameter and 35 mm. long. To this are added 40  $\lambda$  or less of ferricyanide-sodium carbonate reagent and the tube inverted and twirled until the contents are well mixed. Any liquid adhering to the walls may be brought down by use of an air-driven micro centrifuge.<sup>2</sup> The sample is heated on a steam bath for 5 minutes, and then cooled (for this we found convenient a metal disk punched with holes and rotating over a water bath into which the tubes dipped). Just prior to titration, the

<sup>1</sup> 1  $\lambda$  =  $10^{-6}$  liter = 0.001 ml.

<sup>2</sup> All equipment utilized was obtained from the Microchemical Specialties Company, Berkeley, California.

contents of the tube are quantitatively transferred to a porcelain dish by means of a long tipped capillary pipette which is paraffined on the outside. About 50  $\lambda$  of sulfuric acid are added and the contents stirred to release the carbon dioxide. Setopaline C is used as indicator. It is important to add the ceric sulfate from the capillary burette at a reasonably constant rate, or undue variation in titers results. Descriptions of equipment assemblies are found in earlier publications (5, 6).

TABLE III

*Effects of Volume and Pipette Drying on Analysis of Samples Containing 8  $\gamma$  of Glucose*

Final volume of sample	Ceric sulfate, pipette dried in filtered air	Ceric sulfate, pipette rinsed
$\lambda$	$\lambda$	$\lambda$
150	24.19	24.07
200	23.61	24.00
250	24.14	23.90
Mean.....	23.98	23.99
Blank.....	0.93	0.93
	<u>23.05</u>	<u>23.06</u>

### *Sucrose*

To extend the method to analysis of sucrose, the additional point of the procedure required, hydrolysis, may be achieved by use of dilute HCl. Hassid alternatively recommends the use of invertase, but this appears less satisfactory on the microgram scale. The adjustment of pH necessary for optimum enzymic activity is somewhat difficult, and the enzyme itself contributes markedly to the blank titer. In view of the success achieved with acid, we consistently adhered to its use.

*Reagents*—(1) *Hydrochloric acid*, 5 per cent solution. 1 part of concentrated acid to 6 parts of water. (2) *Sodium hydroxide-sodium carbonate solution*. A solution of NaOH is adjusted in concentration until it is isonormal with the HCl. Sodium carbonate is then added to a concentration of 10 per cent.

*Procedure*—The sample of sucrose solution is delivered to a micro test-tube and an equal volume of HCl added. The mixture is then transferred for 10 minutes to a water bath maintained at 69°. With respect to time of hydrolysis we found little difference in values between 5 and 10 minutes. On completion of hydrolysis the tubes are cooled and the contents neutralized with a volume of NaOH-carbonate reagent equal to that of the HCl used. The addition of excess carbonate could not be avoided, since if

omitted, Prussian blue would frequently develop during the oxidation process, presumably because of acidity of the medium. The remainder of the procedure is the same as for reducing sugars.

TABLE IV  
*Recovery of Sucrose*

Sucrose taken	Ceric sulfate (0.0089 N)	Average recovery
7	$\lambda$	$\gamma$
8	24.54	
	24.32	
	24.63	
	24.43	
	24.59	
Average.....	24.51	7.94
6	18.65	
	18.86	
	18.84	
	18.81	
	18.68	
Average.....	18.76	6.08
4	12.17	
	12.40	
	12.40	
Average.....	12.32	3.99
2	6.08	
	6.15	
	6.19	
Average.....	6.14	1.99
1	3.01	
	3.12	
	2.98	
	3.05	
Average.....	3.04	0.99

*Results*—The results obtained by this method are illustrated in Table IV. All readings represent single determinations, so that the extent of possible error in any determination is apparent. Our results would lead us to con-

clude that if the procedure outlined is rigorously followed it is possible to determine 1  $\gamma$  of sucrose with an error of  $\pm 2$  to 3 per cent. The accuracy, of course, increases with the larger quantity.

#### SUMMARY

Further studies of the microgram scale determination of glucose led to improvements in the control of the blank.

The revised procedure was applied to the determination of 1 to 8  $\gamma$  of non-reducing sugar, such as sucrose.

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# MICROGRAM ANALYSIS: A SOLID-LIQUID EXTRACTOR AND ITS APPLICATION TO EXTRACTION OF SUGARS FROM PLANT MATERIALS\*

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(Received for publication, June 18, 1948)

In adapting methods of continuous solid-liquid extraction to the microgram range, the problem is essentially that of efficiently refluxing 75 to 200  $\lambda^1$  of solvent. For this at least two conditions are necessary: The bulk of extracting fluid at any given moment should be in the liquid state, and the condensation should be so arranged to deliver to the extraction cup or thimble sufficiently small droplets without loss to other parts of the vessel. The first of these requirements would hardly be satisfied by most of the micro extractors described by Schneider (1) because of their relatively large size; the second of these becomes troublesome when an attempt is made to scale down the size without modification of design. In general, any type of hollow tube condenser, air- or water-cooled, appears undesirable, since the downward flow of condensate is too frequently obstructed by droplets trapped in the narrow tube by capillary forces. The cold finger type of condenser certainly appears preferable, but even so, the simple expedient of inserting one into a small test-tube is rendered ineffective by the trapping of liquid at various points between walls of condenser and test-tube. We found a large condensing surface within a small volume and with a steep temperature gradient between vessel wall and condenser to be a modification necessary to the successful functioning of a drop scale extractor. Based upon this principle, the extractor described here was designed. With it 75 to 300  $\lambda$  of volatile solvent (alcohol or ether) could be refluxed for several days without appreciable loss in the volume of extraction medium.

In so far as the mechanics of the extractor were concerned, these could be observed and judged by using a variety of solvents. It was desired, however, in view of our interest in the metabolism of pollen mother-cells, to test the apparatus for extraction of microgram quantities of sugars. Also, in order to complete the procedure, we devised and tested means of filtration and evaporation of very small volumes.

\* Aided by grants from the United States Public Health Service, the Research Board of the University of California, and the Royal Society of Canada.

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<sup>1</sup> 1  $\lambda$  =  $10^{-6}$  liter = 0.001 ml.

*Description of Extractor*

The essential features of the apparatus are illustrated in Fig. 1. In its construction it is desirable to make the supporting pegs of minimum diameter. (We achieved this by using a micro torch, fusing a droplet of glass at the desired point, heating the glass locally, and quickly jabbing it with a dissection needle.) It is also advisable to keep the total capacity of the reservoir bulb (including neck) to 400  $\lambda$  or less, if the volume of solvent to be used is of the order of 200  $\lambda$ . Cones of platinum or glass are equally func-

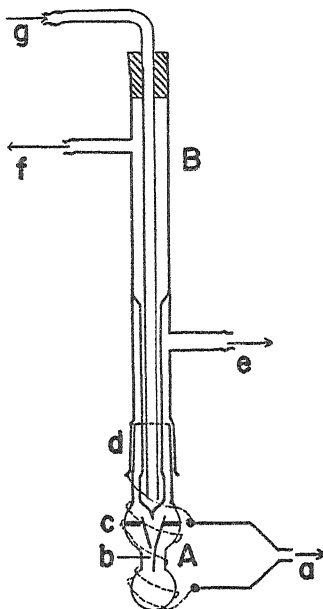


FIG. 1. Microgram extraction apparatus. *A*, extraction vessel; *B*, condenser assembly; *a*, nichrome wire leads connected to variable voltage transformer; *b*, extraction cup fitted with tail; *c*, supporting pegs; *d*, ground glass joint; *e*, air or vacuum outlet; *f*, water outlet; *g*, water inlet.

tional; the tail attachment is necessary for a smooth and directed flow of solvent. The diameter of the condenser bulb should be as large as the neck of the extractor vessel will allow. The height of the air outlet we have not found to be critical.

*Operation of Extractor*

*Heating*—Our experience indicates that the use of nichrome resistance wire attached to a variable voltage transformer is by far the most favorable method of heating. Control of temperature is sensitive and easy. The

current can be adjusted to boil liquid without spattering (we have tried water, alcohol, and ether with equal success) and to any desired rate of droplet condensation. In fact, once the proper voltage is found, adjustments for subsequent extractions are unnecessary. With respect to the nichrome wrapping, it is important that only one or two coils are maintained around the lower bulb, the remainder being distributed over the upper portion of the extraction vessel.

*Extraction Cup*—The arrangement here is arbitrary. We inserted a small cotton plug at the base which served not only to support the tissue but also to control the level of liquid in the cup. By adjusting both plugging and heating it is possible to maintain the liquid in the cup at any desired level. Small filter cones were also tried and they functioned equally well. In both cases, the tail of the extraction cup should be so positioned that the droplets fall directly into the reservoir without hitting the walls. Adjustment, if necessary, is done by gentle tapping of the apparatus.

*Evaporation of Solvent*—This is achieved by halting the water circulation, removing the extraction cup, and attaching the air outlet to a vacuum line. The applied voltage is the same as that used for refluxing. Evaporation in this way is smooth and quick, about 5 minutes being required for total removal of 80 per cent ethanol.

#### *Extraction of Sugars from Onion Root Tip*

To provide experimental material, an onion (*Allium cepa*) was suspended over a beaker of aerated water kept at room temperature and the roots thus formed used in the experiments here described. (Anthers of *Trillium*, for which the research was intended, were unavailable at the time.) Since a physiological study was not being conducted, no attempt was made to control growth conditions. The roots were sliced and their fresh weight determined by a quartz helix balance (2). The portions used were all of the order of 0.3 to 3 mg. As suggested by Hassid (3), 80 per cent ethanol was used for extraction medium and the material refluxed for periods varying from 5 to 24 hours. Maceration of the root appeared unnecessary, since the yields after 6 hours were no less than those obtained after 20 hours of extraction. Following extraction, the alcohol was evaporated down to about 10  $\lambda$  and then transferred, with several washings, to a micro test-tube. It is possible to add reagents directly to the extraction bulb, but this is less convenient when centrifugation is desired. The contents of the test-tube, about 150  $\lambda$  in volume, were again evaporated to 10  $\lambda$ .

Evaporation of solvent from a narrow tube is more difficult than from a bulb with a relatively large surface. It was satisfactorily accomplished, however, by using the arrangement indicated in Fig. 2. The essential point is the use of the nichrome wire to heat the chamber but not the tube.

If a low voltage is first applied and then increased stepwise, evaporation proceeds smoothly and is complete in about 30 minutes. The required voltages need only be established once; evaporations may then be run with little care.

To the 10  $\lambda$  of extract in the tube were added 5  $\lambda$  of saturated neutral lead acetate, and the sides of the vessel carefully rinsed with an additional 5 to 10  $\lambda$  of water. 10  $\lambda$  of saturated disodium phosphate were measured into the mixture, the contents well mixed, and 5 or 10 minutes later centrifuged with the air centrifuge.<sup>2</sup>

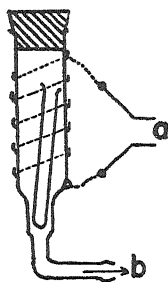


FIG. 2

FIG. 2. Evaporation assembly. *a*, nichrome wire leads to variable voltage transformer; *b*, outlet to vacuum.

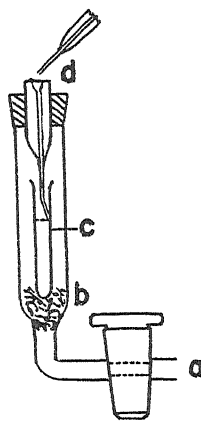


FIG. 3

FIG. 3. Microgram filtration assembly. *a*, vacuum outlet; *b*, supporting glass wool; *c*, calibrated micro tube; *d*, ground glass top of capillary tube on which filter paper rests.

The type of filter we found most suitable is shown in Fig. 3. It is, in fact, generally useful when samples in the neighborhood of 50  $\lambda$  are to be filtered and a quantitative recovery of filtrate is desired. The apparatus is easily made with a short piece of capillary tubing, 1 mm. bore, by enlarging the bore at one end and grinding that end flat. The other end is drawn out and bent slightly at the tip to provide a small point of contact between it and the wall of the calibrated micro tube. It is also advisable to paraffin the lower portion. Suitable filtering disks were obtained by means of a paper punch. In operation, the filter paper was wetted and the suction turned on to assure adhesion of the disk to the ground glass surface. The suction was then stopped and a calibrated micro test-tube

<sup>2</sup> Obtainable from the Microchemical Specialties Company, Berkeley, California.



inserted. A capillary pipette of rather large bore was used to take up successive portions of the mixture which was applied dropwise to the filter disk. The vacuum was so regulated as to provide a smooth flow of filtrate down the side of the tube and no accumulation of liquid on the filter paper. When means of adequate centrifugation are not available, the mixture of precipitate and solution can be filtered directly, but to avoid plugging of the filter, talc is first applied as described by Hassid (3).

The efficiency of this method of clearing was tested with  $\pm \gamma$  samples of glucose. 10  $\lambda$  of glucose solution containing  $\pm \gamma$  of glucose were delivered to a sugar tube, and the reagents added as described above. Controls were simultaneously run in all cases. The results are shown in Table I, where the volume of ceric sulfate required for the filtrate as a percentage of that required for the control is given.

TABLE I  
*Effect of Clearing and Filtration on Glucose Recovery*

Recovery in individual samples expressed as percentage of controls.

Samples centrifuged and filtered	Samples filtered without centrifugation
100.5	99.5
100.1	100.5
101.1	100.4
	98.8

In the case of plant extracts, the filtrate was made up to 200  $\lambda$  and 40  $\lambda$  aliquots used for determination of total sugars and reducing sugars. The size of aliquot is, of course, arbitrary and depends largely upon the total sugar content of the extract. Since even 0.28 mg. of root yielded about 8  $\gamma$  of sugar, it was unnecessary in our case to increase the size of aliquot. A very important point at this stage of the procedure is the mixing of the filtrate once made up to volume. Apparently, inversion and twirling of the tube are inadequate, for quite often if treated this way, the aliquots showed a gradation in concentration, the first one removed always being the lowest. The use of a 200 to 250  $\lambda$  pipette, paraffined on the outside, obviated the difficulty. By successive drawing up and releasing of the solution at different levels in the tube, thorough mixing was achieved.

Again to test for reliability of the method, the recovery of five samples of sucrose was studied (Table II). The variability of the sugar content between different roots and, as would appear from our results, between different portions of the same root precluded the adoption of any particular concentration as a standard. Instead, both these methods were tried: The extract was divided equally, and to one-half, 4  $\gamma$  of sucrose were added,

or, after 6 hours extraction, the extract was similarly divided, one-half was returned to the extraction bulb, 4  $\gamma$  of sucrose added to the extraction cup, and the solution again extracted for a few hours. Both methods yielded similar recoveries.

In view of the successful recovery, some comparisons were made between the sugar content of different roots and between two different portions of the root. The results are listed in Table III. The values are all expressed in terms of sucrose equivalents.

TABLE II  
*Recovery of 4  $\gamma$  of Sucrose in Extracts of Various Sugar Concentrations*

Total sugar content of sample	Recovery
$\gamma$	<i>per cent</i>
94.8	104
78.6	98
25.0	100.2
8.3	101.1
7.6	100.0

TABLE III  
*Sugar Content of Onion Root*

Length of root	Distance of section from tip	Weight of sample	Total sugar per mg. fresh weight	Reducing sugar
<i>cm.</i>	<i>cm.</i>	<i>mg.</i>	$\gamma$	$\gamma$
4	Tip	3.4	23.5	6.5
3	0.5	1.1	17.4	2.9
3	Tip	0.28	28.0	16.8
	1.0	0.90	26.0	6.9
3	Tip	1.73	24.5	5.9
	1.0	1.50	23.1	4.6
1.6	Tip	0.70	21.6	11.7
	0.5	0.89	19.8	2.8

From these results it is apparent that a great deal of variability exists with respect to sugar content. Some of the differences might be real, the higher proportion of reducing sugars at the tip, for example. Others may well be due to variability in water content of the tissues. It is clear, however, that, in so far as the method of extraction is concerned, there are no outstanding differences in recoveries from small or large root sections. This latter point, of course, is our chief interest here.

#### SUMMARY

A microgram extractor, filter, and evaporator are described, as well as the technique necessary in applying this equipment to the analysis of

microgram quantities of sucrose or other non-reducing sugars. The method was tested in the analysis of short lengths of root tips.

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# THE EFFECT OF pH ON THE RESPIRATION OF BRAIN TISSUE; THE pH OF TISSUE SLICES\*

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(Received for publication, July 1, 1948)

With slices of various guinea pig tissues suspended in bicarbonate-free horse serum adjusted to various pH values, Canzanelli *et al.* (1) found that maximum rates of oxygen uptake occurred in all cases in media with pH values well above 7.4. With brain slices, the curve relating respiration rate to the pH of the medium rose with increasing pH, rapidly up to pH 6, less rapidly from 6 to 8, and then rapidly again to give a marked maximum at pH 9 to 9.5. It finally fell sharply with further increase in pH. Canzanelli *et al.* summarized previous work on the effects of pH on tissue respiration. Much of it was unsatisfactory with regard to pH control and measurement and for other reasons. The surprisingly high optimum found by Canzanelli *et al.* suggested that the question should be studied further.

As a result of the studies here reported we conclude that the true optimum pH for the respiration of brain tissue is in the usual physiological range and that the actual pH within slices is usually appreciably different from that of the medium in which they are immersed.

## Methods

Slices of rat brain cortex were prepared in a cool humid chamber (2, 3) by means of a Stadie and Riggs (4) microtome designed to cut slices about 0.43 mm. thick. For respiration measurements the blade was not moistened and the slices were weighed on a torsion balance.

Isotonic suspensions (5) of whole brain were prepared in warm calcium-free Ringer's solution, or in bicarbonate-free horse or sheep serum,<sup>1</sup> containing 0.01 M glucose.

Oxygen uptake was measured by standard procedure in the Barcroft apparatus at 38°. The vessels contained oxygen for slices, air for suspensions. In some experiments samples of medium or suspension were adjusted to various pH levels and 3 ml. were placed in the vessels. More usually each vessel received 2 ml. of suspension or slices and 2 ml. of medium, and 1 ml. of medium containing the amounts of phosphate buffer,

\* An abstract of this paper has appeared (*Federation Proc.*, 6, 249 (1947)).

<sup>1</sup> The serum was not usually inactivated, since in several experiments the respiration rates of slices in inactivated (*i.e.*, heated to 58°) and untreated serum were the same.

NaOH, or HCl, needed to produce the required pH in the mixture was tipped in from a side bulb shortly before zero time. The final concentration of phosphate, when used, was 0.03 M. All mixtures were isotonic. Duplicate vessels were set up. One was removed from the bath at zero time, the other at the end of the experimental period, and the pH of the medium in each was determined immediately with an ordinary glass electrode.

For the measurement of the pH of slices themselves, a small cylindrical stand made of plastic, 25 mm. in diameter, was covered with wide mesh gauze. The slice was laid on this and held in place by a second layer of gauze. These layers were held down by elastic bands around the stand. The stand, with slice and coverings, was lowered into a 50 ml. beaker containing the medium and the latter was continuously aerated with the appropriate gas. The beaker was placed in a larger vessel containing water maintained at about 37°. The pH was measured by means of a micro glass electrode (glass membrane-saturated Ag acetate in 0.5 per cent acetic acid-silver) and reference electrode (cotton wick, normal saline, chlorided silver wire) similar to those described by Nims (6). These were connected to an ordinary Beckman pH meter, the connection to the glass electrode being shielded, and calibrated against buffers of known pH. The electrodes were bound to a flexible bronze strip which was fastened to the arm of a dissecting microscope stand. By means of the ratchet of this stand they could be very gently lowered into contact with the slice or raised to measure the pH of the medium.

### Results

In Fig. 1, *a*, the results of experiments with rat brain cortex slices suspended in bicarbonate-free horse serum are shown. These results confirm those obtained by Canzanelli *et al.* with guinea pig brain in that maximum respiration rates are observed around pH 9. A large number of experiments with slices in Ringer-phosphate-glucose solution indicated that maximum respiration rate occurred at a relatively high pH in this medium, as in serum. However, the pH fell so rapidly in the high range that it was impossible to determine the effect of pH with accuracy.

It has been shown (5) that brain suspensions prepared in isotonic medium respire at a rate comparable with that of slices and present a number of advantages in the study of brain tissue metabolism. The pH of these suspensions can be readily adjusted after homogenization and does not change so greatly as does the medium with slices. Aerobic glycolysis is less active than that of slices (7) and respiratory CO<sub>2</sub> production is lower in the alkaline range, where CO<sub>2</sub> strongly affects pH. Rather dilute suspensions, 30 to 50 mg. of tissue per ml. of final volume, were used to minimize the pH changes.

In Fig. 1, *b*, *c*, and *d*, the relation of the respiration rate of whole brain suspensions in various media to pH is shown. The optimum pH seems to be about 7 to 7.5.

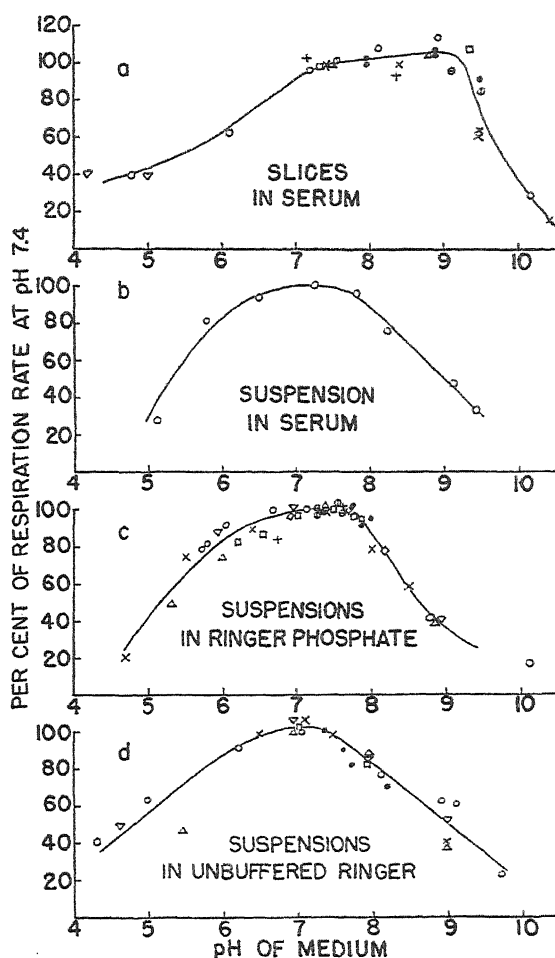


FIG. 1. Effects of pH on the respiration rate of slices of cortex and suspensions of whole brain. Similarly marked points were obtained in experiments on samples of the same tissue. The pH shown was the mean of the values for the beginning and the end of the 30 minute experimental period.

In Fig. 2 the results of experiments are illustrated in which suspensions were adjusted to various pH values, kept at room temperature for various times, and then readjusted to pH 7.4 before the respiration rate was determined. It may be seen that the effects of pH rapidly become irreversible, particularly in the alkaline range.

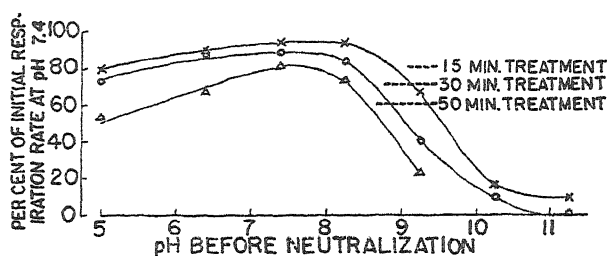


FIG. 2. Effects of keeping suspensions at room temperature in Ringer-phosphate medium at various pH values on the subsequent respiration rate at pH 7.4.

TABLE I  
*pH of Rat Tissue Slices in Various Media*

Medium*	Aeration gas	pH recorded from medium	pH recorded from slice
Brain cortex			
Horse serum†	100% O <sub>2</sub>	8.8	6.7
" "	100% "	6.0	5.9
Ringer-0.03 M phosphate	100% "	8.8	6.5
Same	100% "	8.4	6.6
"	100% "	8.4	7.3‡
"	100% "	8.0	6.5
"	100% "	7.4	6.6
"	100% "	5.8	5.7
"	100% "	4.6	5.0
Ringer-0.02 M bicarbonate	100% "	8.7	7.0
Same	95% O <sub>2</sub> -5% CO <sub>2</sub>	7.4	6.7
Ringer-0.001 M bicarbonate	95% " -5% "	6.0	5.9
Ringer, no bicarbonate	95% " -5% "	4.8	5.3
Liver			
Ringer-0.03 M phosphate	100% O <sub>2</sub>	8.5	7.3‡
Kidney			
Ringer-0.03 M phosphate	100% O <sub>2</sub>	8.4	7.3‡

\* The media contained glucose, except where noted.

† Similar results were obtained in sheep serum.

‡ No glucose in the medium.

While the respiration rate of suspensions is only 40 to 50 per cent of maximum at pH 9, the respiration rate with slices is at its maximum in medium of this pH. This suggests that the pH of the tissue in a slice is not the same as that of the medium in which it is suspended, but is actually



lower and nearer the optimum. That this is true was indicated by the experiments with the micro glass electrode. When electrodes which recorded a pH of nearly 9 in the medium were gently lowered into contact with the slice, the recorded pH immediately fell and, after about 5 minutes, became constant at about 6.6. On raising the electrodes, the original pH of the medium was again recorded. A steady pH value was not recorded immediately when the electrode touched the tissue, presumably because time is required for the fluid within the concavity of the electrode to come

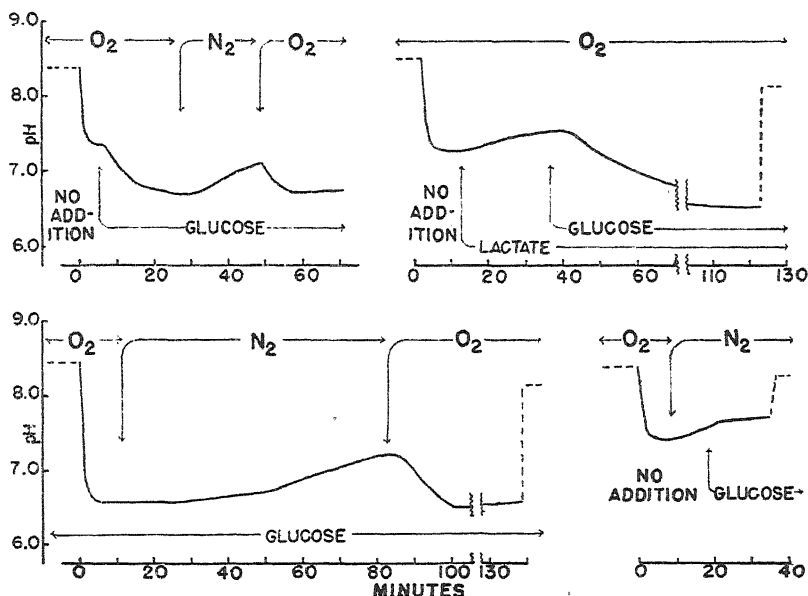


FIG. 3. Changes in pH recorded from brain slices under various conditions. Ringer-0.03 M phosphate medium. Substrates added to the medium and aeration gas changed at times shown by the arrows. Glucose 0.01 M, sodium L-lactate 0.02 M. Solid lines, glass electrode on the slice; broken lines, glass electrode in the medium above the slice.

into equilibrium with that of the tissue. In glucose-containing media of pH above 7, the pH recorded from the slice was 6.5 to 6.7 and seemed usually to be independent of the pH of the medium. In media of lower pH, the pH of the slice fell lower. However, in media of pH below 5, the pH of the slice was found to be appreciably higher than that of the medium.

Similar results (see Table I) were obtained when the medium was serum, Ringer-phosphate, or Ringer-bicarbonate. (Since the volumes of the media were large, their pH did not change greatly.) With liver and kidney cortex

slices similar differences between the pH of the slice and the medium were observed.

With brain slices under aerobic conditions the pH of the slice was appreciably lower with glucose present in the medium than with no substrate or lactate in the medium. With glucose the pH remained constant but with lactate or with no substrate the pH often rose slowly. On changing from aerobic to anaerobic conditions, with glucose present, the pH sometimes showed no immediate change, but after a variable time it rose; the latter trend could be reversed by return to aerobic conditions. In the absence of glucose under anaerobic conditions the pH usually rose, and the rise could be slowed down or arrested, but not reversed, by the addition, after a short time, of glucose. (Dickens and Greville (8) found that brain slices lost metabolic activity very rapidly in the absence of both oxygen and glucose.) It seems evident that the pH of a slice is at least partially dependent upon metabolic activity. Typical records of the above-mentioned observations are shown in Fig. 3.

#### DISCUSSION

The difference in pH between the slice and the medium might be due to the following factors: (a) difference between the carbon dioxide concentrations in the medium and in the inner layers of the slice; (b) difference between the lactic acid concentrations in the medium and in the slice, (c) buffering effect of tissue proteins and other substances; and (d) other factors connected with tissue and cell structure, permeability, and metabolism.

Warburg (9) calculated that a respiring slice of a tissue of thickness approaching the limit for adequate oxygenation of its inner layers would have, at equilibrium, a carbon dioxide tension in its inner layers about 3 per cent of an atmosphere higher than in the surrounding medium. A saline solution buffered with 0.02 M bicarbonate has at 38° a pH of about 9.6 in equilibrium with air and 7.35 with 5 per cent carbon dioxide. An increase of the CO<sub>2</sub> tension by 0.03 atmosphere would lower the pH to 7.5 and 7.1 respectively. If phosphate-buffered solutions, 0.03 M, having initial pH values of 8.0 and 7.4 in air, are equilibrated with 3 per cent CO<sub>2</sub>, it may be calculated that their pH should drop to 7.2 and 7.0 respectively. (The pH of a medium at or below pH 6 would of course scarcely be affected by CO<sub>2</sub>.) Thus the concentration of CO<sub>2</sub> within the slice could theoretically account for a considerable pH difference between the innermost layers of a slice and the medium in the alkaline range under aerobic conditions.

Some determinations were made of the lactic acid concentration in brain slices and in the suspending Ringer-phosphate-glucose medium (pH 7.5) after a period of aeration with oxygen. The slices were rapidly drained and ground up with weighed amounts of sand and 5 per cent trichloroacetic

acid and the mixture reweighed. A sample of the medium was also treated with trichloroacetic acid. Lactic acid was determined on the filtrates by the method of Barker and Summerson (10). The concentrations of lactic acid in the slice and in the medium were found to be about 8 and 3 micro-moles per ml. respectively. The lactic acid concentration within the slice is thus definitely higher than in the surrounding medium and probably contributes to the pH difference, but the extent of this effect cannot readily be assessed. Perhaps the differences in  $\text{CO}_2$  and lactic acid content of the tissue could together account for most of the pH differential of slices respiring in glucose-containing media.

Under anaerobic conditions in the presence of glucose, the pH of the slice was well below that of the medium in spite of the fact that respiratory  $\text{CO}_2$  production would not be contributing to the pH difference. The differential might be accounted for in this case by a large accumulation of lactic acid within the slice.

In the presence of oxygen but in the absence of glucose, aerobic glycolysis would not occur but rather residual lactate within the slice would be consumed. With added lactate there would be definite consumption of lactate with liberation of base. It is thus to be expected that the pH of the slice would be higher in these conditions than in the presence of glucose and oxygen.

It seems probable to us that the buffering effect of the tissue materials must also be concerned. This is probably the main factor which keeps the pH of slices higher than that of the medium when the latter is below 5. Doubtless other factors are also concerned.

The pH of the interstitial fluids within the slice may vary with the distance from the surface and it is uncertain to what portion of the slice the pH recorded by the present method refers. Since the tips of the electrodes were a little less than 1 mm. in diameter and the slice thickness nearly 0.5 mm., it might be suggested that the recorded pH refers to a point nearly 0.5 mm. from the free surface. Since the deepest level in a slice of the usual thickness is half this distance, it is possible that the difference in pH between the medium and the innermost layers of a thin slice is somewhat less than that shown.

Since similar differences in hydrogen ion concentration between the medium and rat brain, kidney, and liver slices have been observed, the phenomenon is presumably general. In most studies on the metabolism of tissue slices in media at about pH 7.4, the actual pH of the interstitial fluids in the slice has evidently been lower than what is commonly considered to be the normal physiological level. But it seems possible that the difference between the pH recorded by an electrode placed on a slice and the pH of the surrounding medium may represent a true physiological

situation and not merely a result of the lack of capillary circulation. This is rendered more probable by *in vivo* observations of Elliott and Jasper (11), who found that the pH recorded from an electrode placed against the pial surface of a lightly anesthetized cat was independent of the pH of fluid irrigating the brain and surrounding the electrode, except its tip, and was always appreciably lower than that of the venous blood. The pH so recorded varied from point to point, depending, apparently, upon the proximity of larger blood vessels, and values as low as 7.0 were obtained.

#### SUMMARY

The true optimum pH for brain tissue respiration is about 7 to 7.5. The pH of slices of brain and other tissues is lower than that of the suspending medium at all pH values above 6.

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# THE METABOLISM OF THIOCYANATE IN THE RAT AND ITS INHIBITION BY PROPYLTHIOURACIL\*

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(Received for publication, July 6, 1948)

In a previous report (1) we have shown that a small fraction of the sulfur of injected thiocyanate is converted to sulfate by the rat. The conversion of thiocyanate to sulfate had been predicted to occur as the result of the physiological activity of thiocyanate ion which is known to be a goitrogen and a hypotensive agent. Our results could not be taken as unequivocal evidence for the metabolism of thiocyanate by the body because, as we have observed, thiocyanate breaks down spontaneously in aqueous solution to yield sulfate. The study did indicate that the processes which might occur in biological systems would involve rather small amounts of thiocyanate ion.

An accumulation of thiocyanate in the adrenals of the rabbit has been suggested (2). Concentration of thiocyanate in the thyroid of the rat (3) has been reported also. In the latter instance the process was likened to a mass action effect. It is well known that thiocyanate interferes with the incorporation of iodine as thyroxine in the thyroid gland (4-6). The chemical similarity between thiocyanate and iodide suggested to us that the antagonism between the two ions could be metabolic. One might expect to find not only free thiocyanate ion in the thyroid fluids but also thiocyanate sulfur bound to protein and in the form of degradation products. We therefore turned to an examination of specific tissues of the animal body for evidence of the products of metabolism of thiocyanate ion.

25 mg. of radioactive potassium thiocyanate labeled with  $S^{35}$  were injected intraperitoneally into rats which were sacrificed at 6 and 24 hour intervals. The whole thyroids and adrenals and samples of blood plasma, muscle, and liver were pooled from groups of six animals. The tissues were homogenized and the protein was precipitated with trichloroacetic acid. The protein precipitates were freed of thiocyanate ion by "washing out" with inactive potassium thiocyanate and then oxidized by peroxide fusion. Radioactivity determinations gave a measure of thiocyanate sulfur bound to protein (Table I).

Free thiocyanate ion was removed from the supernatants of the homogenates by precipitation with silver ion. The thiocyanate was oxidized to sulfate. Radioactivity determinations gave a measure of free thiocyanate in total tissue fluid (Table I).

\* This study was aided by a grant from the Rockefeller Foundation.

$S^{35}$ -containing compounds remained in the liquors after protein-bound and free thiocyanate sulfur had been removed. These were oxidized to sulfate and determined by radioactivity measurements to show the quantity of thiocyanate ion which had been converted to other soluble compounds by the tissues ("rest" thiocyanate sulfur, Table I).

The data in Table I show, as expected, that the major portion of the injected thiocyanate was present after 6 hours as free thiocyanate ion in the extracellular fluid and that the fall in values after 24 hours, as the result of excretion (1), is similar for the total fluids of all tissues. Thyroid, 6 hours after injection, contained free thiocyanate ion in significantly higher concentration than liver, muscle, or adrenal.

Small amounts of thiocyanate sulfur were "bound" to liver, muscle, plasma, and adrenal proteins, while larger amounts were bound to thyroid protein.

In marked contrast to liver, muscle, and adrenal tissue, the thyroid contained conversion products of thiocyanate ion, as indicated by a much larger amount of labeled sulfur in the fraction not precipitated by trichloroacetic acid or silver ion.

We have investigated the effect of *n*-propylthiouracil on the distribution of thiocyanate sulfur in rat tissues. VanderLaan, Bissel, and VanderLaan (4, 5) and Taurog, Chaikoff, and Feller (6) had shown that the uptake of iodide by thyroids of rats treated with propylthiouracil is not inhibited but the iodide remains in unaltered form. The data in Table II show that the administration of propylthiouracil to rats for 33 days before injection of potassium thiocyanate does not stop the uptake of free thiocyanate ion by the gland or the binding of thiocyanate sulfur to the protein. On the other hand, the formation of "rest" thiocyanate sulfur, or metabolized thiocyanate sulfur, is almost entirely repressed.

#### EXPERIMENTAL<sup>1</sup>

White rats were selected in groups of six of the same sex. The weights of individuals of the group generally averaged 230 gm. Each animal was injected intraperitoneally with 1 ml. of solution containing 25 mg. of potassium thiocyanate labeled with radioactive sulfur.<sup>2</sup> Subsequently the animals were maintained on water alone for either 6 or 24 hours. A cardiac puncture was performed under light ether anesthesia. Immediately after-

<sup>1</sup> The authors wish to acknowledge the assistance of Miss Nelson Kingsland who carried out most of the analytical procedures and radioactivity measurements.

<sup>2</sup> Radioactive sulfur was obtained from the Clinton National Laboratory, Oak Ridge, Tennessee, under allocation from the United States Atomic Energy Commission, in separated form as  $Na_2S$ .

ward, the animal was killed by opening the chest. The thyroid, adrenals, and portions of the liver and muscle were removed. Pooled samples of each tissue were ground in a glass homogenizer, first with 1 to 2 ml. of water and again after the further addition of 2 ml. of 10 per cent trichloroacetic acid. The volume was diluted to 10 or 12 ml. with water. The precipitate was separated by centrifugation and the supernatant was transferred to a volumetric flask. 1 mg. of unlabeled potassium thiocyanate was added to the supernatant, and 10 mg. were added to the precipitate, which was stirred up with 10 ml. of 1 per cent trichloroacetic acid and allowed to stand overnight. The supernatant was then added to the first supernatant and the precipitate was washed four times more with 10 ml. portions of 1 per cent trichloroacetic acid. The supernatant liquors from muscle, liver, and plasma respectively were diluted to 100 ml., and aliquots were taken for duplicate sulfur determinations. The adrenal and thyroid liquors were used *in toto*. The pooled plasmas were treated in a similar manner except that 2 per cent trichloroacetic acid was used for washing and a total of 10 mg. of inactive potassium thiocyanate was added to the first supernatant liquor from the homogenate.

"Bound" thiocyanate sulfur was converted to sulfate by fusion of the trichloroacetic acid precipitate with sodium carbonate and peroxide according to the method of Bailey (7). The fusion mixtures were dissolved and made up to 25 ml. in the case of the thyroid and adrenals and to 50 ml. for the liver, muscle, and plasma. The major part of the solution was taken as an aliquot and adjusted to between pH 3 and 4 with cresol red as an indicator. 2 drops of 10 per cent Triton NE wetting agent were added. The sulfate was precipitated from the warm solution with 0.5 ml. of 5 per cent barium chloride. The use of barium (rather than benzidine) for sulfate precipitation was preferred because of the high salt content of the solutions. The precipitate was digested from 2 to 12 hours and then was collected on a disk of No. 42 Whatman filter paper with the candle filter described by Henriques *et al.* (8). The precipitate was washed with dilute hydrochloric acid (1:300) and then with 95 per cent ethanol, and dried in a desiccator over calcium chloride. The average weight of the barium sulfate was 7 mg. Correction was made for self absorption and for radioactive decay by counting a standard sample on the same day. Radioactivity measurements were made with a thin window Geiger-Müller tube.

"Unbound" thiocyanate ion was precipitated from the supernatant liquors as silver thiocyanate. The solution was made alkaline to litmus with ammonium hydroxide, and a saturated solution of silver acetate in 3 N ammonium hydroxide was added until precipitation was complete. The solution was made acid to litmus with acetic acid. The precipitated silver

thiocyanate was collected by centrifugation and washed with 5 ml. of water. The thiocyanate sulfur was oxidized to sulfate with an excess of acid bromate by the method previously described (1).

The sulfate was precipitated with barium ion as described above. Duplicate determinations were carried out on liver, plasma, and muscle.

"Rest" thiocyanate sulfur was determined on the supernatant solutions from the silver thiocyanate precipitate. The excess silver ion was precipitated with dilute hydrochloric acid. The filtrate was neutralized with solid sodium carbonate, then 1 ml. of 10 per cent sodium hydroxide was added, and the mixture was evaporated to dryness. Total sulfate was determined, after peroxide fusion, as described above.

In the case of the supernatant solutions (containing "rest" thiocyanate sulfur) of Rats 42 to 47, 48 to 50, and 54 to 59, 3.5 mg. of sodium sulfate were added to the solution after removal of the chloride. This was followed by the addition of barium chloride solution. The barium sulfate was collected and the radioactivity of the sample was measured. The excess barium in the mother liquor was removed as the carbonate. The mother liquor was then evaporated. This residue was oxidized by sodium peroxide, as described above, for measurement of thiocyanate sulfur which had been converted to some product other than sulfate.

#### DISCUSSION

A definite formation of protein-bound thiocyanate sulfur is seen in a comparison of the values for thyroid with those of plasma, muscle, liver, and adrenals in Table I. It will be noted that proteins of all tissues bound some thiocyanate sulfur. Since thiocyanate ion is *absorbed* by proteins quite strongly *in vitro*, we carried out model experiments to test the reliability of our method for removing free thiocyanate from the trichloroacetic acid precipitate. Labeled thiocyanate was added to the muscle protein, and the precipitation and washing out procedure described in the experimental section were applied. In such tests we could remove 99 per cent or more of the added thiocyanate. In order to assess the effect of stirring in air during homogenization of the tissues we tried aeration of blood plasma in the presence of labeled potassium thiocyanate for 6 hours at 35°. The protein-bound sulfur was not significantly different from that of a non-aerated control solution. We would therefore conclude that no binding of thiocyanate to protein was induced by our analytical procedures and that values for protein-bound thiocyanate sulfur greater than 1 per cent of the total labeled sulfur in the tissue may be considered significant. The thiocyanate sulfur bound to the protein of liver, adrenal, muscle, and plasma may be accounted for either by formation of an actual chemical linkage to protein, by adsorption on protein, or by combination with lipide material as suggested by Lein for iodine (9). The thiocyanate sulfur bound to thyroid and possibly to



TABLE I  
*Content of Free Thiocyanate Ion Sulfur, Thiocyanate Sulfur Bound by Protein, and "Rest" Thiocyanate Sulfur of Tissues of Rats  
 Injected with 25 mg. of Labeled Potassium Thiocyanate (8.33 mg. SCN-S)*

Rat No. and sex	Average weight gm.	Time interval hrs.	Plasma, $\gamma$ S per ml.			Liver, $\gamma$ S per gm.			Muscle, $\gamma$ S per gm.			Adrenals, $\gamma$ S per gm.			Thyroid, $\gamma$ S per gm.		
			Free	Bound	"Rest"	Free	Bound	"Rest"	Free	Bound	"Rest"	Free	Bound	"Rest"	Free	Bound	"Rest"
18-23* ♀	226	6	44	0.16	0.00	19	0.85	0.00	7.8	0.09	0.00	18	1.6	0.00	31	2.0	12
24-29 ♂	230	6	43	0.53	0.00	21	2.3	0.00	7.2	1.5	0.00	15	7.5	0.00	26	17.0	15
30-35 ♂	258	6	48	0.33	0.00	20	0.64	0.15	7.0	0.45	0.03	15	0.21		20	21.0	23
Average.....			45	0.34		20	1.30		7.3	0.68		16	3.1		26	13.0	17
42-47 ♂	253	24	5.2	0.10	0.00	1.4	0.15	0.10	0.64	0.06	0.12	1.1	0.11	0.00	2.6	1.1	35
48-50 ♀	221	24	3.4	0.06	0.00	1.2	0.24	0.22	0.43	0.06	0.07	1.1	0.15	0.00	1.8	2.2	14
54-59 ♀	189	24	4.4	0.05	0.16	1.3	0.16	0.10	0.45	0.01	0.23	1.1	0.45		2.7	4.2	21
Average.....			4.3	0.07		1.3	0.18		0.50	0.04		1.1	0.24		2.4	2.5	23

\* These rats (Nos. 18 to 23) had been received from a supplier more than 1000 miles from Memphis. All other animals were obtained from a local colony.

adrenals could be also the result of formation of chemical compounds analogous to the iodoproteins. The degradation of such unnatural protein derivatives by the body could account for the fall in protein-bound thiocyanate during the 6 to 24 hour period.

The finding of radioactive sulfur in the mother liquors after precipitation of the protein with trichloroacetic acid and precipitation of free thiocyanate with silver was unique with thyroid tissue. This offers the most convincing evidence for the metabolism of thiocyanate by the thyroid gland. The absence of radioactivity in the corresponding residual liquors from the other tissues confirms our estimate of the efficiency of our analytical procedures.

The nature of the metabolic products of thiocyanate to be found in the thyroid is unknown. However, a large portion appears not to be sulfate ion, as judged by the four instances in which we fractionated the "rest" thiocyanate sulfur. Here sulfate ion was added, followed by barium chloride treatment before oxidation. The barium sulfate precipitate carried down from 4 to 45 per cent of the radioactivity of these solutions. Because of coprecipitation known to occur with barium sulfate, this does not indicate that any of the products was sulfate, but it does show that some of the products were *not* sulfate.

The thiocyanate precipitated by ammoniacal silver acetate solution under the conditions employed in these experiments is the free thiocyanate ion present in the cellular and interstitial fluids. The values in Table I thus provide a measure of the ability of the tissue to concentrate thiocyanate ion from the plasma. On an equal weight basis, none of the tissues studied, including the thyroid, shows a higher concentration of thiocyanate than the plasma. This is in contrast to the recognized uptake of iodide by the thyroid. There appears to be no concentration of thiocyanate ion by the thyroid, adrenals, and liver, even if the difference in the water content of the plasma and the other tissues is taken into account. Our data do suggest that free thiocyanate ion penetrated the cells of the glandular tissues. Assuming the same concentration of free thiocyanate ion in the extracellular fluid as in the plasma, it can be calculated from the concentrations found in the various tissues that the liver and adrenals, with interstitial compartments of 28 and 38 per cent respectively (10), must necessarily have some cellular penetration by thiocyanate ion. The muscle, with an interstitial fluid compartment of 15 per cent, cannot have any free thiocyanate ion within the cells. If the thyroid interstitial compartment is estimated to be equal to or less than the adrenal, an intracellular penetration of thyroid greater than that of any of the other tissues has occurred.

The free thiocyanate ion concentration in each of the tissues studied at the 24 hour interval is about one-tenth the 6 hour value. Evidently there is a diffusion of thiocyanate ion into and out of the tissues in equilibrium with the plasma concentration.

Chronic propylthiouracil treatment of rats inhibits thiocyanate metabolism in the thyroid (Table II). An inhibition of the protein-binding phase is reflected by a decrease in protein-bound sulfur from 0.42 micromole per gm. for the normal rat thyroids to 0.08 micromole per gm. for the goitrous glands. This result parallels the effect of the drug on iodide fixation. Iodide held by thyroids of propylthiouracil-treated rats is so loosely bound as to be non-precipitable with trichloroacetic acid, while in normal thyroids all or nearly all is protein-bound (5, 6).

Results of studies with iodine (5, 6, 11-14) suggested the possibility that the administration of propylthiouracil should increase the uptake of thiocyanate by the thyroid. A comparison of the values for normal rats with those of rats treated with propylthiouracil (Table II) shows not an increase but a slight decrease in the amount of free thiocyanate held by the goitrous thyroid after 6 hours.

Thiocyanate ion fulfills the requirements (15, 16) for a specific metabolic antagonist against iodide in thyroxine synthesis. This view-point is supported by many independent data. The resemblance of thiocyanate to iodide in chemical properties is so well known that it is classed as a "pseudo-halogen" (17). The physiological effects of thiocyanate as a goitrogen are reversed by iodide (12, 18). Thiocyanate inhibits the uptake of free iodide by the thyroid gland (4, 5, 11-14, 18-20). This inhibition can be reversed by iodide.

Thiocyanate may antagonize iodide by actually replacing it in the sequence of chemical reactions usually leading to thyroxine synthesis. The first stage in this synthesis is a reversible formation of a loose combination with iodide by the gland (6, 20). This may be a typical example of the substrate-enzyme combination which is usually reversible. This iodide is converted to the protein-bound iodine from which thyroxine eventually arises. Thiocyanate may successfully compete with iodide for the enzyme. This would account for the observed displacement of loosely held iodide by thiocyanate ion (5, 14). To carry the concept further, it seems possible that the thyroid enzyme acts to form a reversible combination with the thiocyanate and maintains this in equilibrium with the plasma. Not all the protein-held thiocyanate is released, however, as the plasma concentration falls. Due to the resemblance in properties between thiocyanate and iodide, the enzymatic reactions proceed to the formation of the chemically bound thiocyanate observed here. Finally, in the sequence of thyroxine-like synthesis, an unnatural thiocyanate derivative meets a block to further enzyme activity.

The action of propylthiouracil supports the concept of metabolism of thiocyanate ion by the thyroid. Analogous to its action on iodine metabolism propylthiouracil treatment does not prevent the uptake of thiocyanate but does suppress protein binding and the formation of other metabolic products.

TABLE II  
*Effect of Propylthiouracil on Distribution of Thiocyanate Sulfur*

Rat No.	Average weight gm.	Plasma, $\gamma$ S per ml.			Liver, $\gamma$ S per gm.			Muscle, $\gamma$ S per gm.			Adrenal, $\gamma$ S per gm.			Thyroid, $\gamma$ S per gm.		
		Free	Bound	"Rest"	Free	Bound	"Rest"	Free	Bound	"Rest"	Free	Bound	"Rest"	Free	Bound	"Rest"
36-38*	133	40	0.25	0.02	12	0.48	0.12	6.5	0.13	0.03	22	0.29	0.15	21	0.72	0.54
39-41*	156	43	0.17	0.02	13	0.30	0.08	4.3	0.19	0.04	15	0.26	0.00	16	0.42	0.47
18-35†	238	45	0.34	0.00	20	1.30	0.05	7.3	0.68	0.01	16	3.1	0.00	26	13	17

\* Female rats made goitrous by feeding 0.1 per cent *n*-propylthiouracil for 33 days; injected with 25 mg. of labeled potassium thiocyanate 6 hours before sacrifice.

† Normal rats; data summarized from 6 hour values in Table I.

## SUMMARY

The distribution of thiocyanate ion and its metabolism products has been studied in rats at 6 and 24 hour intervals after the injection of 25 mg. of potassium thiocyanate labeled with radioactive sulfur.

The protein of the thyroid gland fixes thiocyanate sulfur to a greater extent than the protein of other tissues. This activity is inhibited by propylthiouracil treatment.

An accumulation of water-soluble compounds containing radioactive sulfur was found in the thyroid after 6 hours. The quantity had increased at 24 hours, although free thiocyanate ion and protein-bound thiocyanate sulfur had decreased. The formation of these products was inhibited in rats treated with propylthiouracil. No evidence for the production of these compounds in liver, muscle, adrenals, and plasma was found.

The thyroid tissue maintains no concentration gradient of thiocyanate ion against the plasma, but thiocyanate penetrates the cells of thyroid, adrenals, and liver. The level of thiocyanate ion in all the tissues falls with the fall in the plasma level. Propylthiouracil treatment causes no increase in the thyroid-plasma concentration gradient.

These experimental findings have been interpreted in terms of the metabolic antagonism of thiocyanate for iodide.

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# DECOMPOSITION OF CYSTINE AND WOOL BY TREATMENT IN THE BALL MILL AND AUTOCLAVE

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(Received for publication, July 13, 1948)

In the course of investigation of the microbiological degradation of wool, it is often necessary to treat wool either (a) by steam sterilization at 120° for 15 minutes, (b) by grinding in a ball mill, or (c) by a combination of these. Since we wish to study the degradation by a single microorganism, the sterilization procedure is required. Also, since wool has been shown to be a chemically heterogenous substance both histologically and chemically, grinding was necessary in order to obtain a more uniform substrate and an increase in specific surface for the action of the enzymes liberated by the microorganism. Although it has been known for some time that such treatments induce changes in wool (1-10), the extent and nature of these changes, especially on the insoluble residue, have not been studied in detail. Since we are interested in the changes brought about by the microorganism under study, it is obviously of importance to know the chemical and physical characteristics of wool after being subjected to these treatments.

Routh (7), in studies on wool ground in a ball mill, found little change in the total nitrogen and sulfur content, but the cystine content decreased appreciably. Inorganic sulfates and intermediate oxidation products of cystine were found, and he concluded that the change was oxidative in nature.

Stirm and Rouette (8) make the statement that the moisture content is of the highest importance in the rate of destruction of wool due to heat. Among the decomposition products, they found carbon dioxide, sulfinic acid, alanine, and taurine. They noted that by the exclusion of oxygen the sulfur compounds of wool could not be oxidized to sulfate.

Cohen (9) observed a decrease in the nitrogen content of the water-soluble fraction of wool on successive grindings. He concluded that the production of a water-soluble protein is not primarily dependent on the splitting of the disulfide linkage, but on other linkages in addition to this one. Our own observations support this view.

## EXPERIMENTAL

The wool used had been scoured and defatted, and had a moisture content of 9.9 per cent when in equilibrium with the room, which is kept at

50 per cent relative humidity. Portions of this sample were then either ground in a ball mill or chopped. After these basic treatments, 5 gm. portions of the samples were either heated 1 hour at 110° or autoclaved dry or wet. The chopping process consisted of one passage through a medium sized Wiley mill equipped with a 40 mesh sieve. Autoclaving was carried out at 15 pounds steam pressure (120°) for 20 minutes. A ceramic ball mill containing flint pebbles was used; this mill turned at a rate of 120 R.P.M. Unless otherwise specified, samples were milled for 4½ days or about 800,000 revolutions. All samples were analyzed on a moisture-free basis, having been dried in a vacuum oven at 60° for at least 18 hours. At least duplicate analyses were made in every case.

TABLE I  
*Effect of Grinding and Heat on Wool*

Treatment of fiber	Analysis*		
	Cystine	Sulfur	Nitrogen
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Whole fiber.....	11.5	3.43	16.53
Chopped fiber.....	11.5	3.33	16.36
"    "    heated 1 hr. at 110°.....	10.4	3.16	16.54
"    "    autoclaved dry, 20 min. at 120°.....	8.2	2.90	16.61
"    "    "    wet, 20 "    "    120°.....	8.0	2.59	16.67
Ball-milled fiber.....	8.4	2.84	16.27
"    "    heated 1 hr. at 110°.....	7.5	2.47	16.64
"    "    autoclaved dry, 20 min. at 120°.....	6.5	2.34	16.37
"    "    "    wet, 20 "    "    120°.....	6.6	2.32	16.21

\* Calculated on an ash-free, moisture-free basis.

Total sulfur was determined gravimetrically by the oxygen bomb method and cystine by the photometric method of Kassell and Brand (10). Cysteine was calculated as the difference after blocking the —SH groups by iodoacetic acid (11). The intermediate oxidation products of cystine were sought by the method of Lavine (12) and nitrogen was determined by the micro-Kjeldahl procedure. Qualitatively, sulfhydryl was detected by the nitroprusside reaction, aldehydes by the action of Schiff's reagent, and hydrogen sulfide by lead acetate paper.

### *Results*

The effect of chopping, ball milling, and the subsequent autoclaving on the chemical constitution of wool is summarized in Table I. It is to be noted that these figures represent the composition of the water-insoluble residue. In addition to the changes in cystine, sulfur, and nitrogen, it has



been observed qualitatively that hydrogen sulfide is given off during the grinding process. Previous workers have noted that hydrogen sulfide is given off when wool is autoclaved.

Because a positive nitroprusside test was invariably obtained after ball milling, quantitative determinations for cysteine were made by blocking the  $-SH$  with iodoacetate. In a typical batch of ball-milled wool, the cystine content was 7.52 and the cysteine was 0.48 per cent. Varia-

TABLE II  
*Effect of Grinding on L-Cystine*

Compound	Non-ball-milled cystine		Ball-milled cystine			
	Calculated	Found	Found*			
			Batch 1		Batch 2	
			per cent	per cent loss	per cent	per cent loss
Cystine.....	100.0	100.0	70.1	29.9	80.8	19.2
Cysteine.....	0	0	8.5		5.8	
Sulfur.....	26.68	26.70	24.60	7.9	25.65	4.0
Nitrogen.....	11.66	11.67	10.50	11.1	11.17	4.2

\* Batch 1, about 1,400,000 revolutions; Batch 2, about 1,000,000 revolutions; calculated on an ash-free, moisture-free basis.

TABLE III  
*Qualitative Tests on Wool and on Cystine Ground in Ball Mill*

Material	Test	Result
Water-insoluble residue	Nitroprusside, for $-SH$	Positive
" "	Schiff's reagent, for aldehydes	"
Gases present after milling	Hydrogen sulfide	"
" " " "	Ammonia	"
Water-soluble fraction	Sulfates	"
" "	Intermediate oxidation products of cystine	Negative

tions were within  $\pm 5$  per cent of the above figures. The formation of cysteine was also noted with ball-milled cystine, as shown in Table II.

Since the major change in wool keratin due to the degree of comminution appears to involve the disulfide linkage or cystine content, the effect of grinding on L-cystine was also studied. The results are summarized in Table II.

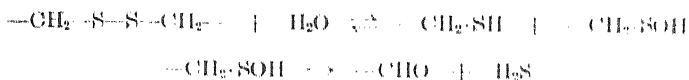
Qualitative tests on both ball-milled wool and cystine gave the results shown in Table III.

## DISCUSSION

The available data are inadequate for a detailed explanation of the mechanism of decomposition of wool or cystine due to grinding. The results, however, suggest generalizations which appear useful in interpreting some of these reactions. That the reactions are complex is indicated by the multiplicity of breakdown products identified, such as cysteine, aldehyde, hydrogen sulfide, ammonia, and sulfates. Although no oxidation products of cystine such as cystine disulfoxide or cysteinesulfinic acid were detected, it does not necessarily follow that they do not appear in the course of the degradation. Other results in these Laboratories indicate that these compounds may appear only momentarily as intermediate steps in the oxidation of cystine to sulfate.

As Cohen (9) suggests, other bonds are broken in addition to the disulfide linkage. This process produces only a partially soluble substance as contrasted to a completely insoluble product obtained from the reduction of wool by means of thioglycolic acid or sodium sulfide. Our own experiences corroborate those of Routh (7) and Cohen (9) that proteolytic enzymes such as pepsin and trypsin readily digest reduced wool and only slowly and partially digest ball-milled wool. This suggests differences in the chemical nature of the two substrates. Possibly the chemical reduction transforms the wool protein to long, insoluble polypeptide chains, whereas ball milling produces small, soluble, dialyzable fragments.

Since it is very difficult to remove all the water from wool, it may be postulated that a hydrolytic cleavage of the disulfide group in wool occurs during the ball milling.



Detailed studies by Schöberl (13) on the above reaction have shown that it occurs with surprising ease. Simple calculations will show that the small amount of water necessary to change the cystine content of wool from 11.5 to 8.4 per cent would be readily available. It is possible that hydrogen sulfide may be oxidized to sulfuric acid, especially in the presence of trace metals (14) which are probably present in the pebbles, accounting in part for the sulfate found present. Although no oxidation products of cystine were actually detected in this study, the further oxidation of sulfinic acid ion to sulfate ion is quite possible. That decrimination of wool or cystine was accelerated by ball milling is partially substantiated by the fact that ammonia was given off and a suspension of either wool or cystine became increasingly acid with increasing the length of time of ball milling. It is known that the impact of pebbles in the rotating mill

generates heat, which may materially catalyze the various decompositions (5). Thus it would appear that during the ball milling both hydrolytic and oxidative processes operate simultaneously.

#### SUMMARY

When either wool or cystine has been subjected to ball milling and auto-claving or either procedure alone, ammonia, hydrogen sulfide, aldehydes, cysteine, and sulfate can be detected. The suggestion is made that these compounds result from simultaneous hydrolytic and oxidative reactions on the wool proteins, the rate of reaction being increased by heat. Quantitative cystine, sulfur, and nitrogen values on wool and cystine under the above conditions support this hypothesis.

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# A FURTHER PURIFICATION OF D-GLUCOSE DEHYDROGENASE\*

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An early preparation of D-glucose dehydrogenase was made by Harrison (1) by the addition of ammonium sulfate to a water extract of acetone-dried mammalian liver. More recently a partial purification was effected by Wainio (2) by fractionation with ammonium sulfate and by the use of aluminum hydroxide gel. The method to be outlined here offers a further purification. Extraction of lyophilized liver with water is followed by adjustment of the pH and by fractionation with ammonium sulfate and calcium phosphate gel. The preparation is completed by removing the last traces of salts by dialysis.

## EXPERIMENTAL

$Ca_3(PO_4)_2$  gel was prepared according to Utkin (3).

*Diphosphopyridine nucleotide* was prepared according to the method of Warburg and Christian (4) as reported by Klein (5).

*Heart flavoprotein* was prepared according to Straub (6) with the slight modification employed by Eichel and Wainio (7).

## Test of Activity

Each fraction was tested for activity by employing the following components in the respirometer vessels: center well, 0.25 ml. of 10 per cent NaOH; side arm, 0.25 ml. of 2 molal D-glucose and 0.25 ml. of diphosphopyridine nucleotide solution containing 1 mg. in 0.25 M  $Na_2HPO_4$ - $KH_2PO_4$  buffer, pH 7.4; body, 0.5 or 1.0 ml. of enzyme solution, 0.5 ml. of Straub's heart flavoprotein solution containing 0.5 mg. in 0.25 M  $Na_2HPO_4$ - $KH_2PO_4$  buffer, pH 7.4, 0.5 ml. of 1:500 methylene blue, and 0.25 or 0.75 ml. of 0.25 M  $Na_2HPO_4$ - $KH_2PO_4$  buffer, pH 7.4. Each fraction was analyzed for total nitrogen by the micro-Kjeldahl method. The results are expressed in terms of  $QO_2$  protein.

\* Aided by a grant from the John and Mary R. Markle Foundation. With the assistance of Herbert J. Eichel.

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*Extraction with Different Media*

The capacity of four different fluids or solutions to extract the dehydrogenase was tested first. Water, 0.1 M NaCl, and 0.1 M  $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ , pH 7.4, gave protein solutions of about the same unit activity, whereas 0.1 M  $\text{CH}_3\text{COOH-CH}_3\text{COONa}$ , pH 4.6, was inferior (Table I). Water proved to be superior to the NaCl solution in that the total active proteins extractable with water were almost 2 times greater than with NaCl.

Three successive extractions were chosen because the third extract had a total activity equal to one-half the total activity of the first and because

TABLE I  
*Fluid Extracts*

Extracting medium	QO <sub>2</sub> protein	Protein ex- tracted mg.	Total activity c.mm. O <sub>2</sub> per hr.
Water.....	21	316	6636
0.1 M $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ , pH 7.4.....	19	166	3054
0.1 M $\text{CH}_3\text{COOH-CH}_3\text{COONa}$ , pH 4.6.....	14	247	3458
0.1 M NaCl.....	20	187	3740

TABLE II  
*(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> Fractions*

(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , saturation per cent	QO <sub>2</sub> protein	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , saturation per cent	QO <sub>2</sub> protein
20-30	46	20-35	101
30-40	20	20-40	77
40-50	5	20-45	60
50-60	2	25-35	115
60-70	0	25-40	78
		25-45	65

the unit activity of the third extract was somewhat greater than the unit activity of the first.

*pH Adjustment*

The pH adjustments were made by lowering the pH to the desired value and, after removal of the precipitate by centrifugation, by readjustment of the pH to 7.0. The pH values tested were those between 5.5 and 5.8, and since they all improved the activity to the same degree, the pH value of 5.7 that Harrison had previously used was preserved for the final preparation.

*Fractionation with  $(\text{NH}_4)_2\text{SO}_4$* 

The addition of  $(\text{NH}_4)_2\text{SO}_4$  in various amounts following adjustment of the pH demonstrated that at 40 per cent saturation most of the active protein was precipitated. A more detailed series of fractionations (Table II) showed that the fraction between 25 and 35 per cent saturation gave the highest unit activity.

*Fractionation with Gels*

A comparison of  $\text{Al}(\text{OH})_3$  and  $\text{Ca}_3(\text{PO}_4)_2$  gels revealed that the latter was superior. Successive adsorptions with the  $\text{Ca}_3(\text{PO}_4)_2$  gel showed that the most active fraction was never the first, but that it might be the second, third, fourth or fifth. Consequently, five successive adsorptions are recommended.

TABLE III  
*Effect of Dialysis*

Preparation No.	Length of dialysis days	$\text{QO}_2$ protein				
		Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5
1	1	46	51	57	132	63
	4				191	426
2	3	54	82	318	90	19
	5	105	170	515		
3	5	79	285	780	870	
4	5			377	704	
5	5			270	360	

*Dialysis*

A final dialysis of 5 days duration is recommended with a change of water each day if no agitating device is available. The last traces of salts must be removed. A marked improvement in activity is noted (from a  $\text{QO}_2$  protein of about 130 to one of 360 to 870) as the time of dialysis is lengthened from 1 day to 5 days (Table III).

*Preparation*

On the 1st day 100 gm. of thoroughly dried lyophilized lamb liver are ground in a mortar to a very fine powder, mixed in the same vessel with 400 ml. of ice-cold distilled water, and extracted in an ice bath for 30 minutes with occasional stirring. The mixture is centrifuged in the cold for 15 minutes at  $2500 \times g$  in 50 ml. celluloid tubes. The supernatant is

decanted through cheese-cloth (the fatty layer held back with a spatula) and the residue reextracted two times with two 200 ml. portions of cold distilled water, with stirring, for 15 minutes. The combined supernatants are dialyzed overnight against distilled water at 5°.

On the 2nd day the solution is centrifuged for 15 minutes at  $2500 \times g$ . The fatty layer, if any forms, is carefully removed and discarded. The pH of the supernatant is usually found to be between 6.0 and 6.5 and is taken to 5.7 with  $N$  HCl. Following centrifugation at  $2500 \times g$  for 15 minutes and subsequent decantation, the supernatant is adjusted to pH 7.0 with  $N$  NaOH. 17.5 gm. of  $(NH_4)_2SO_4$  are added to each 100 ml. of the supernatant to make the latter 25 per cent saturated. The mixture is centrifuged for 15 minutes at  $2500 \times g$  and the precipitate discarded. 7.0 gm. of  $(NH_4)_2SO_4$  are added to each 100 ml. of the supernatant (based on the volume to which the 17.5 gm. per 100 ml. were first added) to make the supernatant 35 per cent saturated. The precipitate is centrifuged (supernatant discarded) and dissolved in 50 ml. of cold distilled water. The proteins are precipitated again by adding 12 gm. of  $(NH_4)_2SO_4$  (35 per cent saturated) and by centrifuging for 15 minutes at  $2500 \times g$ . The precipitate is dissolved in 25 ml. of cold distilled water and dialyzed overnight against distilled water at 5°.

On the 3rd day the solution is centrifuged for 15 minutes at  $2500 \times g$  (precipitate discarded) and mixed with 10 ml. of  $Ca_3(PO_4)_2$  gel (or its equivalent containing 370 mg. of  $Ca_3(PO_4)_2$ ). After standing for 5 minutes in the cold the gel is brought down by centrifuging for 5 minutes at  $1500 \times g$  in 250 ml. glass bottles and saved. The supernatant is successively treated with four more 10 ml. portions of the gel and each gel is set aside and saved. Each of the five gels is eluted with 20 ml. of cold  $0.1 N$   $NH_4OH$  which is 25 per cent saturated with respect to  $(NH_4)_2SO_4$ . After standing in the cold for 5 minutes the gels are once more brought down by centrifuging for 5 minutes at  $1500 \times g$ . Each supernatant is dialyzed against distilled water at 5° for 5 days or until absolutely free of  $(NH_4)_2SO_4$ . A change of distilled water every day facilitates the removal of the  $(NH_4)_2SO_4$ .

On the 8th day, after being centrifuged to remove the copious precipitate, each fraction is tested for activity. The results are expressed in terms of  $QO_2$  protein. The most active fraction ( $QO_2$  protein, 360 to 870) or the two most active fractions, if they do not differ by more than 10 per cent, are lyophilized from the frozen state to yield an active preparation. Lyophilization, however, decreases the  $QO_2$  protein by approximately 50 per cent. The yield from a single fraction is approximately 23 mg.

#### SUMMARY

D-Glucose dehydrogenase has been prepared from lamb liver by the following method: the cells are rendered permeable by lyophilization, the



enzyme is extracted with water, and purification is accomplished by adjustment of the pH and by fractionation with ammonium sulfate and with calcium phosphate gel. Those contaminating proteins that are rendered soluble by small concentrations of salts are removed by a final, complete dialysis. The preparation can be lyophilized, but the unit activity is diminished to the extent of approximately 50 per cent by this treatment.

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# THE PRODUCTION OF RADIOACTIVE CYSTINE BY DIRECT BOMBARDMENT IN THE PILE\*

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(Received for publication, July 28, 1948)

The production of radioactive organic compounds has heretofore required their synthesis from radioactive inorganic building stones. In many cases this is both difficult and costly or even unachievable. We have therefore undertaken the investigation of the possibility of the production of certain radioactive organic compounds by the direct bombardment of the desired compound in the pile. At the outset it should be said that in attempting such an experiment we were not unmindful of the destructive effects caused by the high energies released in the type of neutron capture with which we were dealing. However, it has long been known that the Szilard-Chalmers reaction (13), which makes use of bond fracture following neutron capture, does not go to completion.

We present data here which we believe indicate that this method of production of radioactive organic material is entirely possible. We have chosen cystine with its high sulfur content as our first test material. Bombardment of cystine in the Oak Ridge pile has yielded a sample of cystine containing radioactive sulfur. That the sulfur is not a contaminant has been demonstrated by repeated crystallization of the cystine, by filter paper chromatography, by the formation and recrystallization of cysteine hydrochloride by two different procedures, and by the synthesis of the phenylhydantoin derivative of cystine.

We believe this method of producing radioactive compounds should be applicable to other sulfur-containing compounds, and an experiment with the protein insulin which contains 12 per cent cystine is now under way. Radioactive phosphorus compounds should also be capable of production, and with this end in view, muscle adenylic acid is now also undergoing bombardment.

## EXPERIMENTAL

*Bombardment*—A sample of cystine purchased from the Eastman Kodak Company was recrystallized three times by precipitation at the isoelectric

\* This work was supported in part under a grant from the American Cancer Society recommended by the Committee on Growth of the National Research Council, and in part by the Office of Naval Research.

point. A 2 gm. portion of this cystine was submitted to irradiation for 1 month in the Oak Ridge pile. It was returned in a completely charred condition. A second 2 gm. sample was then sent for 1 month's bombardment in the pile at a position in which both the thermal temperature and the neutron flux were lower. This sample when returned to us, though definitely off white in color, appeared to have suffered relatively little destruction. No actual measurements were made of its total  $S^{36}$  radioactivity. It may be noted that an insoluble residue was present whose gross appearance was similar to S and whose radioactivity was very great. Calculations of the radioactivity retained in the cystine give a total cystine activity of about 5 microcuries, or a specific activity of 0.7 microcurie per mm of cystine. Now, since Overman (8) gives the experimental flux in the pile as  $5 \times 10^{11}$  neutrons per sq. cm. per second ( $\pm 50$  per cent), we may assume, as a first approximation, that the flux for our irradiation was  $10^{11}$  neutrons per sq. cm. per second. Based on this flux, and a cross-section for neutron capture of  $0.011 \times 10^{-24}$  sq. cm. (10), we may calculate that in 1 month's bombardment our cystine sample should have yielded total radioactive S ( $S^{36}$ ) of the order of 7 millicuries. Hence we may calculate that approximately 0.07 per cent of the total radioactive sulfur appeared in the cystine. Calculations of the efficiency of production of radioactive cystine by this method must be accepted as being of a tentative nature, and are given merely as a general guide. There is a probability that a longer bombardment time would increase the yield and the specific activity of the cystine. More accurate information should be forthcoming from the samples now under bombardment.

In addition to sulfur, cystine also contains C, N, H, and O. Only carbon need be seriously considered as a possible radioactive contaminant arising from neutron bombardment, and we expected that the production of  $C^{14}$  would be unimportant compared with that of  $S^{36}$ . Although the cross-section for slow neutron capture by  $C^{13}$  has not been published, an upper limit can be obtained on the assumption that the total cross-section for neutron capture for  $C^{12} + C^{13}$  can be ascribed to  $C^{13}$  alone. This is a most unlikely assumption, but, even so, it yields a cross-section of  $0.0015 \times 10^{-24}$  sq. cm., less than one-fourth that for formation of  $S^{36}$ .

*Counting*—End window counters with windows between 1.5 and 2.5 mg. per sq. cm. thick were used for the measurement of radioactivity. Samples were weighed accurately in approximately 5 mg. quantities (range 4.9 to 5.8 mg.) and distributed as evenly as possible over the surface of the counting cup. To correct for the difference in sensitivity of the various counting tubes used, all samples were measured against a standard of radioactive barium carbonate. No corrections were made for decay, since each sample was measured concomitantly with the cystine from which it was derived

and is so reported in the results. As the experimental work progressed, however, it became evident that the decay of radioactivity was approximately that to be expected for  $S^{35}$ . This decay of radioactivity accounts for the progressive decrease in specific activity encountered in the experiments which will be reported below in the order of their performance. Since the weight of the samples was so uniform, no corrections were made for the difference in self-absorption between samples, because we felt that the uncertainties introduced by the use of different materials for counting was so great that these small corrections would be unimportant (15).

The quality of the radiation was examined by absorbing the radiation in aluminum absorbers. Employing a sample of the recrystallized cystine referred to in the following section as the original stock sample, we found that 96 per cent of the radiation was absorbed by 30.5 mg. per sq. cm. of aluminum, a value in good agreement with that of 31.4 mg. per sq. cm. reported by Solomon, Gould, and Anfinsen (12) for the range of  $S^{35}$  electrons in aluminum. However, this cannot be accepted as conclusive proof that the radioactivity of the sample is due entirely to sulfur, since the range of  $C^{14}$  electrons is 27.8 mg. per sq. cm. Proof that  $C^{14}$  does not contribute any appreciable radioactivity is given in the section on alkaline degradation.

*Recrystallization*—The total bombarded sample was transferred from its container to a lead-protected hood and emptied into a flask containing 50 cc. of  $H_2O$  and 2.5 cc. of concentrated  $HCl$ . The unstoppered flask and its contents were allowed to stand in the ventilated hood for several days to permit any volatile radioactive material to escape. The solution was then filtered and a small insoluble radioactive precipitate rejected. To the yellowish filtrate were then added slowly with shaking 7.5 gm. of sodium acetate  $\cdot 3H_2O$ . A heavy precipitate formed and the mixture was allowed to stand overnight in the hood. The next day the precipitate was centrifuged, washed three successive times, and the precipitate air-dried in the hood. The supernatant and washings have been stored, but no investigations have been performed upon them. The dry precipitate weighed 1.584 gm., and if assumed to be all cystine, represents 79 per cent of the sample sent for bombardment. Its radioactivity was 3250 counts per minute per mg. The sample was again dissolved in dilute  $HCl$  and an insoluble residue centrifuged. This residue had the appearance of sulfur. It was washed with water and dried. Its total weight was 7.2 mg. and it was so radioactive that no direct count was possible. The cystine was again precipitated by the addition of sodium acetate, and the precipitate washed repeatedly with water and immediately redissolved in dilute  $HCl$ . No insoluble residue was encountered this time and the cystine was precipitated from solution for the third time by sodium acetate. After wash-

ing and drying, the three times recrystallized sample gave a count of 1200 per minute per mg.

A 100 mg. portion of the radioactive sample was now mixed with 1.9 gm. of pure cystine and subjected to a variety of recrystallization procedures. The first step employed was to dissolve the cystine mixture in 50 cc. of dilute HCl and to boil it for 10 minutes with 1 gm. of acid-washed charcoal. The material recovered by precipitation at the isoelectric point after this treatment had a count which could not be lowered significantly by any number of subsequent recrystallizations. These included three consecutive charcoal treatments and recrystallizations, the count per mg. on the initial sample being 24.9 per minute, and after the third recrystallization 21.3 per minute. In another series SnS was used as a scavenger.  $\text{SnCl}_2$  was added to the acid cystine solution in an amount equivalent to the cystine present. The tin was precipitated by bubbling in  $\text{H}_2\text{S}$ , and the cystine remaining in the supernatant precipitated, washed, and dried. The process was repeated, yielding a small sample of cystine with a count of 21 per minute per mg. It may be calculated from the radioactivity of these samples that a similar purification of the main sample should yield material with counts lying in the range of 420 to 498 per mg. per minute.

The original undiluted sample of cystine was now treated with charcoal and recrystallized twice more. A total of 0.86 gm. of pure white crystalline material was obtained which gave a count of 465 per minute per mg. Calculated on the basis of cystine, this corresponds to 112,000 counts per mm per minute. We calculate that at the time of its removal from the pile this cystine would have had a count of 235,000 per mm per minute if a decay rate equal to that of  $\text{S}^{35}$  is assumed.

This sample of radioactive cystine was assumed to be as pure as could be achieved by recrystallization procedures, and it was employed for the subsequent experiments. It will be referred to as the original stock sample.

*Migration on Filter Paper*—Proof for the fact that the radioactivity of our cystine sample was not due to a contaminant was next sought by means of filter paper chromatography (5). We have used both phenol and butyric acid as solvents and employed a capillary ascent procedure similar to that described by Williams and Kirby (14). The cystine samples were dissolved in 0.05 N HCl to give a concentration of 2 mg. per cc., and 0.01 cc. was applied to the paper. The original stock sample of radioactive cystine was employed so that 0.01 cc. of solution containing 20  $\gamma$  of cystine should give a total count of approximately 9 per minute. For comparison, a three times recrystallized sample of ordinary cystine was employed. At the end of the run, the portion of the paper containing the control sample of cystine was developed with ninhydrin and  $R_F$  values for its migration were obtained which agreed with

those reported in the literature (5, 14). The portion containing the radioactive sample was marked along the line of expected migration with circles 4.5 cm. in diameter, overlapping each other by 50 per cent, and the radioactivity of successive circles counted. Radioactivity was found to be present chiefly at a position corresponding to the location of the migrated cystine in the control sample. Some radioactivity was also found at the initial point of application of the radioactive cystine sample. Development of the paper with ninhydrin after the counts had been made indicated the presence of cystine only in that area corresponding to the migrated radioactivity. The result can be considered as qualitative in nature only, owing to the low total count per unit area counted. More rigid quantitative proof of the presence of the radioactivity in the cystine molecule itself was therefore sought.

*Formation of Cysteine*—Since a sulfur atom seemed the most likely source of radioactivity in our sample, we have prepared cysteine from our radioactive cystine by two different procedures. The sample of cystine employed for this purpose was a mixture of 1 part of our stock radioactive sample with 4 parts of three times recrystallized normal cystine. A total of 500 mg. of this mixture was dissolved in 20 cc. of dilute HCl and the solution well mixed. A 2 cc. sample was then withdrawn and the cystine precipitated by the addition of sodium acetate. The sample, after being washed three times with water and drying, weighed 45.2 mg. It served as the radioactive cystine sample with which the cysteine samples to be described were compared.

*Tin Reduction*—To 9 cc. of the cystine solution in a small Erlenmeyer flask were added 450 mg. of tin-foil and 3.5 cc. of concentrated HCl. The air in the flask was displaced with CO<sub>2</sub> and the mixture gently rocked overnight at 38°. The unchanged Sn was removed and H<sub>2</sub>S gas passed in until all the tin sulfide was precipitated. This precipitate was centrifuged and excess H<sub>2</sub>S displaced by a stream of CO<sub>2</sub>. The clear solution remaining was taken to dryness *in vacuo* over CaCl<sub>2</sub> and KOH pellets. The white, crystalline, dry residue weighed 274 mg. It was extracted with 5 cc. of hot absolute ethyl alcohol and a small insoluble residue was discarded. The clear alcohol solution was then cooled and stored in the cold overnight. A small crop of crystals formed and was centrifuged and dried *in vacuo*. Their total weight was 12.2 mg. A 5.4 mg. sample was then weighed and counted and yielded 52.2 counts per minute per mg. The total available material was then pooled and weighed 11.4 mg. When dissolved in 1 N HCl and titrated with the aid of a micro burette, it required 1.70 cc. of 0.0392 N iodine. The molecular weight of the material may be calculated as 171. The theoretical molecular weight for cysteine·HCl·H<sub>2</sub>O is 175.5. On the basis of a molecular weight of 171, the count per mm per minute

is 9750. The sample of cystine derived from the solution employed for this experiment gave a count of 20,100 per mm per minute, or 10,050 per 0.5 mm of cystine. The cystine obtained thus has 97 per cent of the theoretical count.

*Electrolytic Reduction*—The remaining 9.0 cc. of cystine solution described in the preliminary paragraph of this section were diluted to a volume of 250 cc. and subjected to electrolytic reduction. The procedure employed was similar to that described by Andrews (2). When titration with iodine showed that the theoretical amount of cysteine had been formed, the solution was removed and evaporated to dryness *in vacuo* over  $\text{CaCl}_2$  and KOH pellets. The residue was poorly soluble in hot alcohol and appeared to be largely in the form of cysteine. It was dissolved readily by dilute HCl, but no precipitate formed on addition of sodium acetate to bring the pH to 4.6. A portion of the acetate solution was titrated with iodine and a white crystalline precipitate of cystine immediately appeared. This was centrifuged, washed three times with  $\text{H}_2\text{O}$ , and dried *in vacuo*. It gave a count of 16,950 per mm per minute. The original cystine from which it was derived showed a count of 19,750. To the remainder of the acetate solution of cysteine, a 5 per cent solution of  $\text{HgCl}_2$  was added until no further precipitate occurred. The white precipitate was centrifuged and washed three times with  $\text{H}_2\text{O}$ . It was then dissolved in 15 cc. of 1 N HCl, a small insoluble portion centrifuged, and the clear solution treated with  $\text{H}_2\text{S}$ . The precipitate of  $\text{HgS}$  was centrifuged and the supernatant freed from  $\text{H}_2\text{S}$  with  $\text{CO}_2$ , filtered, and evaporated to dryness *in vacuo* over  $\text{CaCl}_2$  and KOH pellets. The white crystalline residue was dissolved in the minimum amount of hot absolute ethyl alcohol and the clear hot solution cooled slowly and then allowed to stand overnight in the cold room. The crystalline precipitate was centrifuged in the cold and dried *in vacuo* over paraffin and  $\text{CaCl}_2$ . Each of two 10 mg. samples dissolved in 1 N HCl required exactly 1.40 cc. of 0.0392 N iodine for its oxidation. The calculated molecular weight from these data is 182. On this basis the radioactivity of this sample of cysteine  $\cdot \text{HCl} \cdot \text{H}_2\text{O}$  was 7050 counts per mm per minute. The cystine from which it was derived, counted at the same time, gave 8650 counts per 0.5 mm per minute. This sample of cysteine thus had 82 per cent of the theoretical count.

*Cystine Phenylhydantoin*—Additional evidence for the occurrence of the radioactivity in the cystine molecule itself has been sought by preparing the phenylhydantoin derivative. Preparation of this derivative involves reactions with both the amino and carboxyl groups of cystine and is therefore exceptionally useful for the purpose at hand. The sample of cystine employed was a 1:4 mixture of our stock radioactive sample and normal cystine. It was prepared by solution of the mixture in dilute HCl and precipitation with sodium acetate.



The phenylhydantoin was prepared according to the directions given by Patten (9) and Shiple and Sherwin (11). A 100 mg. sample of cystine was dissolved in 1.2 cc. of  $H_2O$  and 0.9 cc. of 1.0 N NaOH. Then 0.1 cc. of phenyl isocyanate was added with shaking and the mixture shaken intermittently at room temperature for 10 minutes. 1.0 N HCl was next added to neutrality, with the formation of a white gelatinous precipitate. Distilled water (20 cc.) was added and the precipitate broken up with a stirring rod and centrifuged. The precipitate was again washed with 20 cc. of water. The precipitate was next dissolved in 5 cc. of acetone and centrifuged to remove a small amount of insoluble material. By the slow addition of 5.0 cc. of water a precipitate was formed which was centrifuged and washed three times with water. Now 25 cc. of 10 per cent HCl were added and the mixture heated on the steam bath for 2 hours. On cooling, a heavy crop of crystals separated. These were centrifuged and washed into a sintered glass crucible, washed with water, and air-dried. Hot 95 per cent ethyl alcohol was then drawn through the crucible to dissolve the hydantoin, and on cooling a heavy crop of white crystals formed. These were centrifuged and washed twice with alcohol at the centrifuge and dried *in vacuo*.

The radioactivity of the phenylhydantoin derivative was 12,680 counts per minute per mm. The cystine sample from which this derivative was prepared gave 13,370 counts per minute per mm. The derivative thus shows 94 per cent of the radioactivity of the cystine from which it was prepared.

*Alkaline Degradation*—Our final step has been to determine the atom or atoms in the cystine molecule responsible for its radioactivity. As mentioned earlier, the sulfur atom is the most likely possibility, but the carbon atoms cannot be completely excluded. We have therefore subjected a sample to alkaline degradation in the manner employed by Clarke and Inouye (4). In this method the sulfur is fixed as lead sulfide and the carbon chain, which transiently appears as pyruvic acid, can be trapped by formation of a hydrazone. The sample of cystine employed was the same as that described for the preparation of the phenylhydantoin derivative.

*Procedure*—To a 100 mg. sample of cystine, 291.5 mg. of lead acetate ( $3H_2O$ ), 300 mg. of phenylhydrazine hydrochloride, 5.5 cc. of 1.0 N NaOH, and a drop of butyl alcohol were added and the mixture refluxed for 29 hours. The precipitate of PbS was washed with 0.1 N NaOH until the washings were colorless, converted to sulfate, and precipitated as  $BaSO_4$ . The  $BaSO_4$  precipitate was washed three times at the centrifuge with water, resuspended in water, and plated for counting. The  $BaSO_4$  had a count of 7650 per minute per mm. The cystine from which it was obtained had a count of 13,370 per minute per mm, or 6685 per atom of sulfur. The activity of the  $BaSO_4$  precipitate therefore accounts for more than the total

activity of the cystine and may be explained by a greater "internal reflection" (cf. Yankwich and Weigl (15)) of the sulfur radioactivity in the presence of barium.

The supernatant from the PbS precipitate was filtered and acidified with glacial acetic acid and allowed to stand in the cold overnight. The precipitate formed was filtered on a sintered glass crucible and washed with a little  $\text{H}_2\text{O}$ . Hot alcohol was then drawn through the crucible and the clear, dark yellow solution stored in the cold overnight. The next day the crystals that had formed were centrifuged, washed once with a little cold alcohol, and dried *in vacuo*. They showed no radioactivity, indicating that the carbon chain of the cystine molecule is not responsible for the radioactivity of the sample.

#### DISCUSSION

It can be calculated, with the atomic masses given by Livingston and Bethe (7) and the equations of Edwards and Davies (6), that the  $\gamma$ -ray given off as a consequence of the formation of  $\text{S}^{35}$  has an energy of 5.4 m.e.v. The recoil energy of the  $\text{S}^{35}$  nucleus is 447 electron volts, corresponding to 10,400 kilocalories per mole, an energy much greater than that of normal chemical bonds. Since we now know that in spite of this disproportionate energy it is possible to retain some  $\text{S}^{35}$  in the cystine, we may examine the mechanisms which may account for this. Burton (3) and Allen (1) in recent reviews of the subject put forward the "cage effect," a back reaction dependent upon the inability of the radioactive nucleus to escape from the crystal lattice. The  $\text{S}^{35}$  may either fall back into the space which it has left behind it, or penetrate to some other nearby similar space. In the case of bombardment in solution, Burton further suggests that excited molecules may lose their excitation by collision before they have time to dissociate. With excess energy in the amount of 447 electron volts, this process is not very likely. None the less, experiments should be undertaken to compare the relative efficiency of production of radioactive compounds by bombardment as solids and in solution. Burton points out that in larger molecules, as for example insulin, the recoil energy may be distributed among the many available bonds and as a consequence the probability of nuclear synthesis may increase.

Edwards and Davies (6) have suggested that if the  $\gamma$  radiation following neutron capture is given off by more than 1 quantum the recoil energy may be considerably lower than the figure of 447 electron volts. Indeed, should 2 quanta be given off in opposite directions, the  $\text{S}^{35}$  recoil energy could become zero. If such a mechanism is responsible for the production of radioactive cystine, then the low yield we have obtained indicates that the occurrence of such a reaction is relatively infrequent.

We wish to express our thanks to Mr. R. R. Edwards for his many valuable suggestions, and to Mrs. Wallace MacDonald for measuring the radioactive samples.

#### SUMMARY

1. A sample of cystine bombarded in the Oak Ridge pile has been shown to become radioactive.

2. Repeated crystallization of the sample yielded a product with a constant specific activity of 112,000 counts per mm per minute.

3. Migration of samples on filter paper with phenol and butyric acid as solvents showed that the radioactivity migrated qualitatively with the cystine.

4. Samples have been reduced to cysteine by metallic tin and also electrolytically. The cysteine hydrochloride obtained by reduction with tin was recrystallized from alcohol and possessed 98 per cent of the theoretical radioactivity. The cysteine produced electrolytically was precipitated as the Hg salt, freed from mercury with  $\text{H}_2\text{S}$ , and the cysteine hydrochloride obtained recrystallized finally from alcohol. It possessed 82 per cent of the expected radioactivity.

5. The phenylhydantoin derivative of the radioactive sample was prepared and found to contain 94 per cent of the radioactivity expected.

6. Samples of the radioactive cystine were subjected to alkaline degradation in a manner so as to trap the S as PbS and the carbon skeleton as pyruvate hydrazone. The PbS was converted to  $\text{SO}_4$  and precipitated as  $\text{BaSO}_4$ . Its radioactivity accounted for all the activity of the cystine sample. The hydrazone was not radioactive.

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# THE EFFECT OF GROWTH AND ADRENOCORTICOTROPIC HORMONES ON THE AMINO ACID LEVELS IN THE PLASMA\*

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(Received for publication, July 28, 1948)

It has generally been assumed that changes in the level of the free amino acids in the plasma may be caused by factors influencing protein metabolism. It would therefore be expected that the anterior hypophyseal growth and adrenocorticotrophic hormones would influence the concentration of free amino acid in the blood stream, for it is well established that these two hormones have definite effects on protein metabolism (1). It will be seen in the following that growth hormone causes a reduction in the free amino acid plasma level in normal and hypophysectomized rats, while adrenocorticotrophic hormone induces an opposite effect.

*Experiments with Normal Rats*—Male rats 40 days of age were fasted 18 hours before injections. Different dosages of hormone preparations were administered intraperitoneally and the animals were sacrificed 6 hours later. The animals were anesthetized with sodium amytal and blood was taken from the inferior vena cava with heparin as an anticoagulant. The plasma amino acid content was determined by the gasometric ninhydrin reaction (2). The growth and adrenocorticotrophic hormones were isolated from ox and sheep pituitaries respectively by methods previously described (3, 4).

The results obtained with growth hormone are summarized in Table I. When 1.0 mg. of the hormone was injected 6 hours before blood samples were taken, the free amino acid plasma nitrogen from ten rats was found to have decreased from an average of 5.43 to 4.30 mg. per 100 cc. of plasma. This change was highly significant statistically as computed by the method of Fisher (5). Indeed, when blood samples were taken 3 hours after the injection, instead of the usual 6 hours, the lowering of the amino acid nitrogen was already evident, the level being 4.66 mg. per 100 cc. of plasma.

Two other dosages of growth hormone were tested. When 0.5 mg. of the hormone was administered, the lowering of the plasma amino acid nitrogen was not significant, although the change was in the same direction. At a higher dosage (3.0 mg.), the effect was almost identical with that obtained

\* Aided by grants from the American Cancer Society (recommended by the Committee on Growth of the National Research Council), the United States Public Health Service, RG-409, and the Research Board of the University of California, Berkeley.

at the 1.0 mg. dose level. When the animals were killed 12 hours after the injection, the amino acid level had returned to the control level. Finally, it may be emphasized that injections with an inert protein (bovine albumin) did not change the concentration of plasma free amino acids.

Similar experiments with adrenocorticotrophic hormone are summarized in Table II. When 3.0 mg. of the hormone were injected 6 hours prior to death, an elevation of the free amino acid nitrogen was found, an elevation from the control value of 5.43 to 6.07 mg. in 100 cc. of plasma; the change was not, however, highly significant statistically. When rats were injected intraperitoneally thrice daily for 4 days with a total daily dose of

TABLE I

*Effect of Growth Hormone on Plasma Amino Acid Nitrogen of Normal Rats*  
Male rats 40 days old were fasted 18 hours before injections.

Dosage	No. of rats	Body weight at death	Plasma amino acid nitrogen	p*
mg.		gm.	mg. per 100 cc.	
0.0	10	132.6 $\pm$ 4.1†	5.43 $\pm$ 0.23	
0.0‡	6	128.7 $\pm$ 4.1	5.77 $\pm$ 0.21	<0.30
0.5	6	132.3 $\pm$ 1.9	4.89 $\pm$ 0.24	0.15
1.0	10	128.2 $\pm$ 5.6	4.30 $\pm$ 0.14	<0.001
1.0§	5	141.4 $\pm$ 8.0	4.66 $\pm$ 0.21	<0.05
1.0	6	135.4 $\pm$ 3.5	5.23 $\pm$ 0.47	
3.0	5	128.8 $\pm$ 9.2	4.49 $\pm$ 0.25	0.03
3.0	6	121.7 $\pm$ 1.4	5.35 $\pm$ 0.33	<0.7

\* Random probability of difference between injected group and control; see Fisher (5).

† Mean  $\pm$  standard deviation.

‡ Injection of 3 mg. of bovine albumin instead of hormone.

§ Killed 3 hours after the injection instead of the usual 6 hours.

|| Killed 12 hours after injection.

5.0 mg. of the hormone, the levels of plasma amino acid nitrogen increased significantly from 5.43 to 7.37 mg. per cent.

Thus, it is clear that growth hormone causes a reduction in the free amino acid level in the plasma, whereas adrenocorticotrophic hormone increases it.

*Experiments with Hypophysectomized Rats*—Male rats were hypophysectomized at 40 days of age, and 14 days were allowed to elapse before they were used. All animals were fasted 24 hours before death; intraperitoneal injections of hormone solutions were carried out 3 or 6 hours before blood samples were taken. It may be seen in Table III that there is a definite lowering of the plasma free amino acid level in 3 hours with 1.0 mg. of growth hormone. If blood samples were taken at 6 hours, the free amino

TABLE II

*Effect of Adrenocorticotrophic Hormone on Plasma Amino Acid Nitrogen of Normal Rats*

Experiment	No. of rats	Body weight at death	Free amino acid nitrogen	<i>p</i> *
		<i>gm.</i>	<i>mg. per 100 cc. plasma</i>	
A†.....	6	121.0 ± 3.2†	6.07 ± 0.20	0.09
B§.....	5	114.0 ± 2.6	7.37 ± 0.33	<0.001
Control.....	10	132.6 ± 4.1	5.43 ± 0.23	

\* Random probability of difference between injected group and control; see Fisher (5).

† Male rats, 40 days of age, were fasted 18 hours and then injected intraperitoneally with 3.0 mg. of hormone; killed 6 hours subsequently.

‡ Mean ± standard deviation.

§ Male rats, 39 days of age, were injected intraperitoneally thrice daily for 4 days with a total daily dose of 5.0 mg. of hormone; the animals were fasted for 24 hours before death on the 5th day.

TABLE III

*Effect of Growth Hormone on Plasma Amino Acid Nitrogen of Hypophysectomized Rats\**

Dosage	Time of injection prior to death	No. of rats	Body weight at death	Amino acid nitrogen
<i>mg.</i>	<i>hrs.</i>		<i>gm.</i>	<i>mg. per 100 cc. plasma</i>
0.0	0	10	113.7 ± 8.1†	4.01 ± 0.14
1.0	3	5	101.2 ± 1.7	3.51 ± 0.07
1.0	6	4	108.3 ± 2.0	5.06 ± 0.17

\* Male rats were hypophysectomized at 40 days of age; after 14 days the animals were fasted 18 hours before injections.

† Mean ± standard deviation.

TABLE IV

*Effect of Adrenocorticotrophic Hormone on Plasma Amino Acid Nitrogen of Hypophysectomized Rats*

Experiment	No. of rats	Body weight at death	Free amino acid nitrogen
		<i>gm.</i>	<i>mg. per 100 cc. plasma</i>
A*.....	6	108.8 ± 1.5†	4.43 ± 0.26
B†.....	9	116.4 ± 7.1	4.77 ± 0.15
Control.....	10	113.7 ± 8.1	4.01 ± 0.14

\* Hypophysectomized male rats, 40 days of age at operation and 14 days post-operative, were injected with 3 mg. of hormone 6 hours prior to death; the animals were fasted for 18 hours before the injections.

† Mean ± standard deviation.

‡ Injections began on the day of hypophysectomy with 0.2 mg. of hormone daily for 14 days; the animals were fasted 24 hours before death.

acid level would have returned to a value higher than that found in the control.

Table IV presents a summary of the data obtained with adrenocorticotrophic hormone. When 3 mg. of the hormone were injected 6 hours prior to death, the free amino acid level in the plasma was somewhat increased, though not significantly statistically. On the other hand, if the hormone (0.2 mg. daily) was injected on the day of hypophysectomy and for 14 days thereafter so as to cause adrenal hypertrophy, the concentration of free amino acid nitrogen was significantly elevated from 4.01 to 4.77 mg. per 100 cc. of plasma.

It should be noted that hypophysectomy itself produces a significant lowering of the plasma amino acid.

#### DISCUSSION

The influence of the pituitary on the non-protein nitrogen constituents of the blood has been studied by various investigators. Teel and Watkins (6), working with normal dogs, reported a drop of 20 to 30 per cent in the blood non-protein nitrogen after injections of growth extracts, and showed that about 70 per cent of the decrease in non-protein nitrogen was accounted for by decreases in the amino acids and urea. Gaebler (7), also with dogs, confirmed these findings, and in addition found a decrease in urinary nitrogen chiefly at the expense of the urea. A transient fall in plasma amino acids in dogs has also been reported to occur following intraperitoneal injections of a growth-promoting pituitary extract (8).

Experiments with rats have given similar results; Harrison and Long (9) have reported that injections of anterior pituitary extracts into fasted rats caused a decrease of the blood non-protein nitrogen. Fraenkel-Conrat *et al.* (10) found a fall of blood amino acids in both normal and hypophysectomized rats after injections of a purified growth hormone preparation. Recently Frame and Russell (11) found that the administration of whole anterior pituitary extract decreased the blood amino acid in normal rats but had no effect on eviscerated animals.

All the changes which have been cited were assumed to be caused by the growth hormone, although rigidly purified growth hormone preparations had not been employed. The results now reported show clearly that the growth hormone is indeed responsible for reduction in the plasma amino acid level. In normal rats, the effect occurs within 6 hours and disappears after 12 hours; 1.0 mg. of the hormone seems to be an optimal dosage for maximal lowering. In hypophysectomized rats the effect is not as evident as that obtained in normal animals. The amino acid concentration in the plasma of hypophysectomized rats is already at a very low level and further lowering of this level appears to be attended with difficulty.



The fact that growth hormone reduces the blood amino acids seems justifiably to be brought into relation with its effect on protein anabolism. Together with other findings, *e.g.* that growth hormone causes a marked retention of nitrogen and an increase in the protein content of the carcass of treated animals, the hormone may be regarded as a protein anabolic agent (1). If this is true, the contrasting results obtained with adrenocorticotrophic hormone are not surprising. Evidence so far accumulated has indicated that adrenocorticotrophic hormone is a specific growth-inhibiting agent (1). For instance, adrenocorticotrophic hormone enhances the urinary excretion of nitrogen and potassium in rats (12) and counteracts growth-promoting action of growth hormone (13). It is therefore to be expected that adrenocorticotrophic hormone elevates the concentration of free amino acid in the plasma. This may be due either to a direct protein catabolic action of the hormone or an inhibition of the synthesis of tissue protein, subjects which need further investigation.

#### SUMMARY

Administration of the anterior hypophyseal growth hormone causes a significant decrease in the blood amino acid content in normal and hypophysectomized rats, whereas the adrenocorticotrophic hormone elevates the blood amino acid level.

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## THE LIBERATION OF PANTOTHENIC ACID FROM COENZYME A\*

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(Received for publication, July 31, 1948)

It was reported previously that coenzyme A contained pantothenic acid bound in such a manner as to make it unavailable in microbiological tests (1). A liberation of  $\beta$ -alanine on acid hydrolysis indicated early the presence of pantothenic acid, which was confirmed by chick assay (2). While the chick assay for pantothenic acid and the  $\beta$ -alanine assay by the yeast growth test yielded equivalent amounts of pantothenic acid, the microbiological assay for pantothenic acid with *Lactobacillus arabinosus* was practically negative with the intact coenzyme. The problem arose, therefore, how to liberate pantothenic acid from the coenzyme.

Since pantothenic acid itself is very sensitive to any severe treatment with strong acid or alkali, the use of enzymatic methods to free the vitamin seemed most hopeful. It had been observed variously that the coenzyme activity was destroyed enzymatically. It appeared probable that such destruction may be due to the removal from pantothenic acid of attached groups which were essential for coenzyme activity, but, on the other hand, inhibitory to the utilization of the vitamin by intact microorganisms. A very rapid inactivation had been found with preparations of intestinal phosphatase (3) as well as with the pigeon liver extract used in acetylation experiments (4). Inactivation by intestinal phosphatase was accompanied by a complete liberation of organically bound phosphate. Inactivation by the pigeon liver extract, however, proved not to be due to a removal of phosphate but to a so far undetermined reaction. A combination of the two agents eventually proved necessary for a complete removal from pantothenic acid of all attached groups. After the incubation of coenzyme A with intestinal phosphatase and liver extract, the microbiological assay of the coenzyme checked well with the results obtained by chick assay and by the  $\beta$ -alanine test with acid-hydrolyzed coenzyme. Only little capacity to liberate pantothenic acid in coenzyme was found with clarase, mylase, and prostate phosphatase.

### *Enzyme Preparations*

*Liver Extract*—The extract is routinely prepared from acetone powder of pigeon liver as described by Kaplan and Lipmann (5). Chicken liver may

\* This work was aided by a grant from the Commonwealth Fund.

be used equally well, but very little activity was found with rabbit liver extract. The enzyme responsible for the liberation of pantothenic acid is relatively unstable. Cooling is, therefore, necessary during the extraction and centrifugation. The inactivation of coenzyme as described in the assay method has to be omitted because standing at room temperature destroys the enzyme rapidly. The enzyme is, however, perfectly stable in the frozen state and is preserved in a deep freeze for continued use. The extracts used in these experiments were prepared by rubbing acetone powder into 10 times its weight of 0.02 M bicarbonate solution with outside cooling. The final extract is obtained by centrifugation for 15 minutes at high speed in the cold.

*Intestinal Phosphatase*—The procedure of Schmidt and Thannhauser (6) is followed essentially. Dr. Gerhard Schmidt has been kind enough to make available to us such modifications as were applied more recently: Digestion of the intestinal extract with trypsin and toluene at 37° is now continued for 1 to 2 weeks, instead of 24 hours, the toluene being renewed frequently. Ammonium sulfate precipitation is then carried out as described, followed by overnight dialysis against ammonium acetate-ammonia buffer. This enzyme solution contains very little pantothenic acid and was found suitable for our experiments. Trypsin does not interfere, and it is, therefore, unnecessary to remove it for our purpose.

A number of experiments were carried out with a highly purified preparation kindly given to us by Dr. Gerhard Schmidt.

Recently a dry preparation of intestinal phosphatase was prepared at Armour and Company in Chicago, which corresponds in activity to Schmidt and Thannhauser's crude phosphatase. It was made available to us in generous quantities and has been used successfully in our tests. It contains only negligible amounts of pantothenic acid. A 2 per cent solution of the dry powder was used, containing 20 to 30 units (6) per ml.

Intestinal phosphatase is strongly inhibited by phosphate (6). Therefore, phosphate may not be used as buffer, and the concentration of phosphate in the sample should be kept as low as possible. We buffer our solutions with bicarbonate, adding enough to bring the pH to 8 to 8.5, which is near enough to the somewhat more alkaline pH optimum of the intestinal phosphatase.

#### *Enzymatic Inactivation of Coenzyme A and Liberation of Pantothenic Acid*

Preliminary experiments had shown that neither intestinal phosphatase nor liver extract alone would satisfactorily liberate pantothenic acid, although both agents completely inactivated the coenzyme. This finding complicated the problem but offered, on the other hand, some clues to the constitution of the coenzyme. It appeared that at least two different

linkages had to be broken to free pantothenic acid of attached groups which prevented independently its utilization as a vitamin in microbiological tests. The chemical aspects of the problem will be discussed elsewhere.

*Separate and Combined Action of Two Enzymatic Principles*—Details of the pantothenic acid assay of enzymatically treated coenzyme A will follow in the paragraph describing the method of pantothenic acid liberation even-

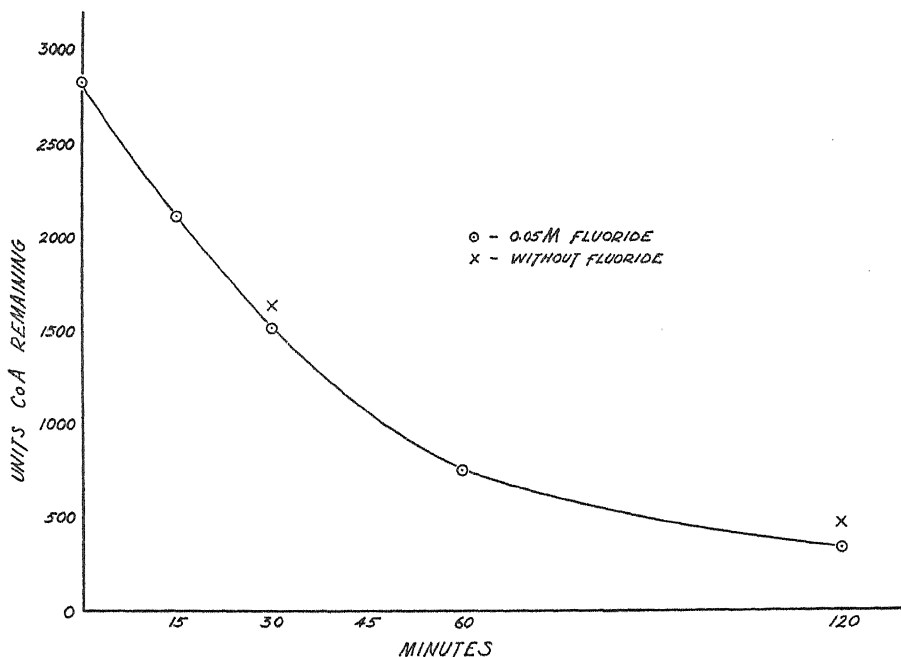


Fig. 1. Destruction of coenzyme A by fresh pigeon liver extract. The reaction mixture contained 10 ml. of liver extract and 2800 units of coenzyme A in a final volume of 67 ml.; incubated at 37°. A relatively large amount of liver extract was used in this experiment to match the amount of coenzyme A used. Aliquots were taken for assay.

tually adopted. The microbiological response is used in the following experiments as a measure of free pantothenic acid.

*Effect of Liver Extract*—The autolytic inactivation by liver extract of a heat-stable factor necessary for sulfanilamide acetylation was observed early in the study of enzymatic acetylation (4). This observation led eventually to the isolation and partial identification of coenzyme A. Some data on the inactivation were included in earlier publications, particularly in the recent description of the method for coenzyme A estimation (5). The gradual destruction of added coenzyme A with time is shown in Fig. 1,

from which it appears that it is a relatively slow process. In our routine procedure, a 3 hour period of incubation was found necessary. The data of Fig. 1 show, further, that the addition of fluoride does not prevent coenzyme A inactivation. Fluoride, however, prevents practically completely the liberation of phosphate by the liver extract. In Table I liberation of phosphate and of pantothenic acid in the presence and absence of fluoride is compared with inactivation with variously treated enzyme preparations.

TABLE I  
*Effect of Pigeon Liver Extract*

Coenzyme A preparations of about 100 units per mg. were used, containing approximately 9 per cent phosphorus and 8 to 10 per cent pantothenic acid.

	Coenzyme A inactivation	Phosphate* release	Pantothenic acid liberation
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Fresh extract.....	90-100	25-50	30-50
" " + 0.05 M NaF.....	90-100	0	0
Dialyzed extract.....	25	25	25-30
" " + 0.05 M NaF.....	0	0	0
Aged extract.....	25-50		15-20

\* Phosphate was measured by the method of Fiske and Subbarow (13).

TABLE II  
*Effect of Intestinal Phosphatase on Release of Inorganic Phosphate and Pantothenic Acid from Coenzyme A*

The composition of the coenzyme A preparations was similar to those in Table I.

Phosphatase* added	Phosphate liberated	Coenzyme A inactivation	Pantothenic acid liberated
<i>ml.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
0.05	90	100	25
0.10	98	100	28
0.15	98	100	28
0.20	100	100	30

\* The highly purified preparation of Dr. Schmidt was used. It contained 50 units per ml. The samples were incubated for 3 hours at 37°.

A considerable loss of activity with dialysis and aging indicates the instability of the inactivating liver enzyme. This emphasizes its non-identity with phosphatase, which is quite stable.

*Effect of Intestinal Phosphatase*—In a manner analogous to that with the liver enzyme, the functional inactivation with phosphatase is compared with liberation of phosphate and pantothenic acid in Table II. The data show that increase of enzyme concentration above a certain level does not

increase markedly the liberation of pantothenic acid; even at the highest level not more than 30 per cent of the total pantothenic acid becomes available by dephosphorylation of coenzyme A. The phosphatase used in these experiments was a highly purified preparation, and could not have contained more than traces of other enzymes. It is suggested by this observation that in the microbiological assay the product of dephosphorylation of coenzyme A is only one-third as active as free pantothenic acid.

*Combined Action of Two Enzymes*—The data with the two enzymes separately indicated that a mixture of phosphatase and liver might liberate pantothenic acid completely. Liver extract alone had given up to 50 per

TABLE III

*Comparison of Various Preparations of Liver Enzyme for Release of Pantothenic Acid*

Intestinal phosphatase 0.25 ml.; other preparations 0.05 ml.; pH 8.5. Total volume 1.5 ml. 3 hours incubation at 37°.

	Liberation of pantothenate	
	Total	Excess due to liver enzyme
	<i>per cent</i>	<i>per cent</i>
Intestinal phosphatase alone.....	20	
+ fresh pigeon liver enzyme.....	90	70
+ aged pigeon liver extract (4 hrs., room temperature).....	20	0
+ dialyzed pigeon liver extract (24 hrs., 5°).....	30	10
+ 70% ammonium sulfate fraction of fresh pigeon liver extract.....	39	19
+ 42% ammonium sulfate fraction of fresh pigeon liver extract.....	35	15
+ rabbit liver extract.....	25	5
+ chicken " ".....	92	72

cent liberation. Such liberation of pantothenic acid was, however, abolished by the addition of fluoride which suppressed phosphate liberation. Therefore, combinations of intestinal phosphatase and liver enzyme were tested systematically. The results of such experiments are shown in Table III. The excess liberation due to liver extract measures its activity towards the residual linkage in the coenzyme. After inactivation of the liver extract by aging or dialysis, neither addition of boiled fresh extract nor of cysteine restored activity. Some fractionation with ammonium sulfate was tried but not very successfully. It appears, furthermore, from Table III that pigeon and chicken liver are about equally active; rabbit, hog, and beef liver were only slightly active.

The results of the experiments discussed are summarized in Table IV which shows that, with suitable amounts of the two enzymes, the full equivalent of pantothenic acid appears after treatment. This observation suggested a method for pantothenic acid liberation in coenzyme-containing material.

### Procedure

In mapping out the present procedure, consideration had to be given to the relatively large pantothenic acid blank introduced by one of the enzyme solutions. It has been mentioned that the intestinal phosphatase preparation, fortunately, contained only negligible amounts of pantothenic acid. This is not true, however, for the liver enzyme. The 0.05 ml. of fresh pigeon liver routinely used has a content of 0.5 to 1.5  $\gamma$  of pantothenic

TABLE IV  
*Summary of Action of Liver Extracts and Intestinal Phosphatase*

	Coenzyme A inactivation	Phosphate liberated	Pantothenic acid liberated
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Fresh liver extract.....	90-100	25-50	30-50
“ “ “ + 0.05 M NaF.....	90-100	0	0
Intestinal phosphatase.....	100	100	20-30
Fresh liver extract + intestinal phosphatase.....	100	100	85-95

acid, mostly in the bound form. The optimum region for the microbiological assay is 0.02 to 0.2  $\gamma$ .

With a base-line of about 1  $\gamma$ , the sample to be assayed should contain not much less than 5  $\gamma$  of total pantothenic acid. In an emergency it was found possible, however, to determine 1 to 2  $\gamma$  with reasonable accuracy. The final dilution for the microbiological assay, then, has to be 100- to 250-fold. The high dilution of the sample for the eventual microbiological test has the advantage of minimizing turbidities in tissue extracts which otherwise might interfere with the nephelometric determination of the growth response.

*Pantothenic Acid Assay of Coenzyme A-Containing Material*—For the liberation of bound pantothenic acid, 0.05 ml. of fresh pigeon liver extract, 0.2 ml. of intestinal phosphatase (= 4 to 6 units (6)), and 0.05 ml. of 0.12 M sodium bicarbonate were added to the sample. 5 ml. volumetric flasks were used. The volume was uniformly adjusted with water to 1.3 (1 to 1.5) ml., and the flasks, including the blank with enzymes, were incubated



for 3 hours in a water bath at 37°. At the end of the incubation period, water was added to the 5 ml. mark. To bring the pantothenic acid content within the range of the microbiological assay the samples were then further diluted (see the third column, Table V). The diluted samples were tested at two levels, 1.0 ml. and 2.0 ml., and the results of the two levels were averaged. Table V gives a detailed example of the results of such an experiment.

The pantothenic acid is measured by the method of Skeggs and Wright (7), with the medium of Cheldelin *et al.* (8), with *Lactobacillus arabinosus* 17-5 at two levels in duplicate. The individual samples for the microbiological assay were prepared as described by the authors and autoclaved for 10 minutes at 120° before inoculation with the organism. We found that coenzyme A is not destroyed by autoclaving at neutral

TABLE V  
*Assay of Total and Free Pantothenic Acid*

Assay No.	Boiled rabbit liver extract	Dilution for microbiological test	Pantothenic acid			
			Per ml. diluted sample*	Dilution factor	Per extract sample	Per ml. liver extract†
	ml.		γ	γ	γ	γ
1	0.2	1:12.5	0.065	66.5	4.3	21.5
2	0.4	1:25	0.063	133	8.4	21.0
3	0.5	1:25	0.088	133	11.7	23.3
4	0.7	1:50	0.057	265	15.3	21.8
5	0.9	1:50	0.072	265	19.1	21.3
6	Blank	1:25	0.010	133	1.3	

\* Corrected for blank.

† Free pantothenic acid 0.7 γ per ml. of liver extract (determined by direct microbiological assay with 0.1 ml. of liver sample, undiluted).

or slightly alkaline reaction. The readings were made, after 16 to 20 hours incubation, with a Klett colorimeter. The pantothenic acid values were read from a standard curve run with each assay.

Free pantothenic acid is determined by direct microbiological assay on the sample at convenient levels.

#### *Comparison of Coenzyme Function and Pantothenic Acid Content in Tissues*

The main purpose of the experiments reported in this paragraph was to determine how large a part of tissue pantothenic acid was bound in coenzyme A. Boiled tissue extracts were prepared as described previously (5), and particular care was taken to avoid autolysis before boiling the tissue. The procedure of enzymatic liberation was then used on such tissue extracts for pantothenic acid determination.

The experiment of Table V, which was presented as an example for the procedure, was carried out with samples of boiled liver extract and likewise belongs with this series. It showed that, in the fresh tissue, only 3.3 per cent of the total pantothenic acid was present in free form. The fact that so large a quantity of pantothenic acid was liberated with the method developed for pantothenic acid determination in coenzyme A indicated that pantothenic acid in liver was present mostly, if not entirely, bound in coenzyme A. An even more convincing test was possible by comparison of the pantothenic acid values by microbiological assay with coenzyme activity expressed in our units. It was reported previously (1) that coenzyme A preparations contained, independent of purity, 0.65  $\gamma$  of pantothenic acid per unit.

TABLE VI

*Relation of Coenzyme A Activity and Pantothenic Acid Content in Organs of Rabbit*

All values are given per gm. of wet weight of tissue.

	Coenzyme A	Pantothenic acid			
		Free	Total	Bound	
				Found	Calculated*
	units	$\gamma$	$\gamma$	$\gamma$	$\gamma$
Liver.....	112	1.2	75	74	73
Heart.....	26.4	3.3	20.7	17	17
Kidney.....	49.5	2.7	45	42	32
Brain (cortex).....	40.5	3.0	18	15	26
Testes.....	25.6	6.0	20.4	14	17
Muscle (skeletal).....	6	5.1	9.9	5	4

\* Calculated by multiplication of the unit value by 0.65, the average pantothenic acid content in micrograms of a unit of coenzyme A.

Experiments were carried out, therefore, to compare coenzyme activity and pantothenic acid content in various tissues. The data obtained are reproduced in Table VI. Again free pantothenic acid generally is only a small fraction of the total pantothenic acid. Only muscle appears to contain a larger quantity, about 50 per cent, of free pantothenic acid. In the two last columns of Table VI bound pantothenic acid is compared with coenzyme units. The pantothenic acid equivalent of the unit values is obtained by multiplying by 0.65, the average pantothenic acid content in micrograms of a unit of coenzyme A. The correspondence between pantothenic acid bound as calculated and determined is reasonably good. Only brain shows a lower value of bound pantothenic acid than would be expected from unit determination. This may be due to a considerable tur-

bidity blank in the case of the brain, even with high dilutions, in the microbiological assay.

The same correspondence of liberated pantothenic acid and coenzyme A content is found in bacteria. In Table VII such data are similarly arranged; again the calculated and determined values for pantothenic acid corresponded very closely. Some of such data had been mentioned in an earlier publication (9).

A further proof for a binding of most or all of the pantothenic acid in coenzyme A is given in the experiment represented in Table VIII. Here

TABLE VII  
*Pantothenic Acid-Coenzyme A Relationship in Some Representative Bacteria*

	Coenzyme A	Pantothenic acid			
		Free	Total	Bound	
				Found	Calculated*
	<i>units per gm.</i>	$\gamma$	$\gamma$	$\gamma$	$\gamma$
<i>Lactobacillus arabinosus</i> (deficient).....	54	7	40	33	35
Propionic acid bacteria.....	410	52	330	263	268

\* From coenzyme A unit value, as indicated in Table VI.

TABLE VIII  
*Changes of Coenzyme A Activity and Free Pantothenic Acid during Liver Autolysis*  
The values are given per gm. of fresh tissue.

	Coenzyme A	Free pantothenic acid	Liberated pantothenic acid Coenzyme A disappearing
	<i>units</i>	$\gamma$	
Fresh.....	45	0	
After autolysis.....	12	22.5	
	-33	+22.5	22.5/33 = 0.68

free pantothenic acid and coenzyme A activities were compared in fresh and autolyzed liver. A liberation of pantothenic acid by liver autolysis was very early recognized by Williams (10). In our experiment the liver was removed as fast as possible after the animals were killed, and a sample was taken at once and immediately boiled. The rest of the liver was then incubated in a moist chamber for 3 hours at 37°, and samples were taken after autolysis. Determination of the coenzyme activity before and after autolysis shows that 73 per cent of the coenzyme was destroyed. The disappearance of coenzyme was accompanied by an appearance of considerable

amounts of free pantothenic acid. A closer comparison of coenzyme A which disappeared and liberated pantothenic acid gives an indication how much of the latter had been derived from coenzyme A. It is shown in the last column of Table VIII that 0.68  $\gamma$  of pantothenic acid was liberated for every unit of coenzyme A disappearing, while 0.65  $\gamma$  of pantothenic acid had been found per unit of isolated coenzyme A.

#### DISCUSSION

In a recent review of the microbiological assay of pantothenic acid, Jukes (11) summarizes the previous efforts to liberate the vitamin in natural materials and to make it available to the microorganism. Particularly with yeast, liver, and similar sources there remained the impression that the problem was never solved satisfactorily. The microbiological return of pantothenic acid never reached the levels obtained in the chick test whatever enzyme or enzyme mixture was used for its liberation. It was suggested that the results may indicate a linked form of pantothenic acid which was not available in the microbiological assay. During our recent work with coenzyme A, we became very well acquainted with a compound which contained pantothenic acid bound in just such a manner. The pantothenic acid bound in intact coenzyme A appeared practically unavailable to living microorganisms (1). In the chick test, however, the coenzyme returned most or all its pantothenic acid (2). In the attempts to liberate, for microbiological assay, pantothenic acid from coenzyme A, we inadvertently obtained, at the same time, a reliable method for microbiological determination of pantothenic acid. This is indicated in the results reported here, but it appears still more clearly in a comparative study by Neilands and Strong (12) on a great variety of food materials, comparing the yield of pantothenic acid by the earlier mylase method (older treatment) and by our present enzyme mixture. Although, against untreated samples, mylase treatment increased the yields, with the mixture of liver enzyme and intestinal phosphatase up to 4 times more pantothenic acid appeared, particularly from animal materials. These results are in accord with our experience on the treatment of coenzyme A with mylase or clarase. We find that very little, if any, pantothenic acid was liberated from intact coenzyme. If coenzyme A, however, had already undergone preliminary autolysis, clarase and mylase tended to split out more pantothenic acid. As will be reported in more detail elsewhere, fragments of coenzyme A may be attacked by mylase and clarase, while the intact coenzyme is stable with these enzymes.

These observations already indicated that a large fraction of tissue pantothenic acid was bound in coenzyme A. It is well known that some vitamins appear in a variety of coenzymes, while others seem to be present in

only one metabolically active form. It appeared, therefore, important to explore how far pantothenic acid could be identified quantitatively with coenzyme A. For this purpose values for the coenzyme unit were compared with those for the pantothenic acid by use of the previously reported factor of 0.65  $\gamma$  of pantothenic acid per unit of coenzyme A. The practical identity of calculated and found values of bound pantothenic acid in liver and the general close correspondence indicate that pantothenic acid appears in living cells largely, maybe only, in one metabolically active form.

#### SUMMARY

1. The enzymatic inactivation of coenzyme A is compared with the liberation of pantothenic acid and inorganic phosphate; with intestinal phosphatase only about one-third of the pantothenic acid is liberated with complete dephosphorylation and inactivation. With pigeon liver extract complete inactivation is parallel with partial liberation of pantothenic acid and phosphate; phosphate, as well as pantothenic acid, liberation may be suppressed by fluoride without effect on functional inactivation of the coenzyme.

2. A mixture of intestinal phosphatase and fresh pigeon liver extract liberates practically all of the pantothenic acid from coenzyme A, and makes it available for microbiological assay. A procedure is described for microbiological determination of pantothenic acid in coenzyme A-containing material with this enzyme mixture.

3. A close correspondence is found between the determined pantothenic acid content and the pantothenic acid equivalent of coenzyme A activity for animal tissues and other living cells. It is concluded that most, possibly all, cellular pantothenic acid is bound in coenzyme A.

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# THE APPLICATION OF PAPER PARTITION CHROMATOGRAPHY TO STEROID ANALYSIS

## I. KETOSTEROIDS

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Paper partition chromatography, originally devised by Consden, Gordon, and Martin (1) for separation of amino acid mixtures, has been adapted previously for several other classes of compounds (2-10). The method depends upon differences in the relative solubilities of compounds in the components of a two phase solvent system. One phase, stationary, is formed by water bound to the cellulose fibers of ordinary filter paper. The other, moving, is an organic solvent immiscible with water. It is allowed to move by capillarity along the paper past a point upon which the mixture to be analyzed has been placed. Each compound moves to a characteristic position revealed by a color reaction performed after the development and drying of the chromatogram.

Certain properties of the steroids render analysis by this method difficult. Some of them have extremely low solubilities in water and they do not lend themselves readily to the production of colors with non-corrosive reagents. In a preliminary report (11) we have indicated that these obstacles can be overcome in the case of many ketosteroids by formation of hydrazones with Girard's Reagent T (12) prior to chromatography. In this manner the water solubilities of the compounds are greatly augmented, and highly reactive quaternary nitrogen groups are made available for the production of colors with alkaloid reagents. Isomeric differences cannot be detected by this method and, furthermore, certain carbonyl groups do not seem to form stable hydrazones.

### *Materials---*

1. *Ketosteroids*<sup>1</sup> were used as supplied without further purification.
2. *Filter paper*. Whatman No. 1.
3. *Girard's Reagent T* (trimethyl acet-hydrazide ammonium chloride) was obtained from the Eastman Kodak Company. It was purified periodically by recrystallization from absolute ethanol and stored in a desiccator.

<sup>1</sup> Crystalline ketosteroids were generously supplied by Dr. C. D. Kochakian of the Department of Physiology and by Dr. E. Oppenheimer and Dr. C. R. Scholz of Ciba Pharmaceutical Products, Inc.

4. *Absolute methanol* was prepared as follows: Baker's c.p. absolute methanol was allowed to stand in contact with magnesium foil for 24 to 48 hours. The methanol was then distilled off and stored in a glass-stoppered bottle.

5. *Methanolic acetic acid*. A solution of glacial acetic acid, 10 per cent by volume, in absolute methanol was prepared at weekly intervals.

6. *n-Butanol*, acid-free redistilled.

7. *Potassium iodoplatinate*. 5 ml. of 5 per cent platinum chloride in 1 N hydrochloric acid, 45 ml. of 10 per cent potassium iodide, and 100 ml. of distilled water were combined, and the solution was stored in a dark, glass-stoppered bottle.

8. *Kraut-Dragendorff reagent*. A solution consisting of 4 gm. of bismuth subnitrate in 23.5 ml. of nitric acid (specific gravity 1.18) was added slowly, with constant stirring, to a solution of 13.6 gm. of potassium iodide in 25 ml. of water. The orange-red solution was placed in the cold for 2 hours. Precipitated crystals of potassium nitrate were filtered off and the filtrate diluted to 200 ml. with water. The solution was allowed to stand for 30 minutes. The fine black precipitate which appeared was removed by centrifugation. The clear orange supernatant was stored in the dark.

9. *Tollens' reagent*. Equal volumes of 0.1 N silver nitrate and 5 N ammonium hydroxide were mixed shortly before use.

#### *Formation of Hydrazones with Girard's Reagent T*

*Method A*—A convenient quantity of ketosteroid (usually 2.5 to 5.0 mg.), twice this weight of Girard's Reagent T, and 2 ml. of the alcoholic acetic acid solution were introduced into an all-glass refluxing apparatus protected against moisture by a tube containing anhydrous calcium sulfate. The solution was refluxed over steam for  $\frac{1}{2}$  to 1 hour and after cooling was stored in a well stoppered vial in a refrigerator. It was found in model experiments with androsterone that 93 per cent of the steroid was converted to the hydrazone by this method. This was determined by estimating the steroid content of the so called "ketonic" fraction by a modification<sup>2</sup> of the method of Langstroth and Talbot (13). The derivative was stable even at room temperature for at least 2 weeks.

*Method B*—An alternative method, in which the ketosteroid, Girard's Reagent T, and acidified methanol were incubated at 40° for 2 hours, was used more recently.

Separate samples of androsterone, testosterone,  $\Delta^4$ -androstenedione-3,17, and pregnanol-3( $\alpha$ )-one-20 were converted to hydrazones by each of the two methods. Aliquots of the individual preparations corresponding to 25,

<sup>2</sup> 95 per cent ethanol (aldehyde-free) was used to dissolve the steroid, *m*-dinitrobenzene, and potassium hydroxide, and 10 ml. of 80 per cent ethanol were used to dilute the reaction mixture after incubation for 60 minutes.



15, 10, and 5  $\gamma$  of steroid were then run side by side on the same chromatogram. It was found that samples prepared by this alternative method gave spots of the same size and intensity as equivalents prepared as described for Method A. More precise quantitative evaluation of Method B has not been made. The conversion of labile ketosteroids (for example, those having an  $\alpha,\beta$ -unsaturated ketone group) to their corresponding Reagent T hydrazones should be carried out in a nitrogen atmosphere with Method B.

### *Chromatography*

Filter paper strips  $6 \times 45$  cm. or sheets  $28 \times 40$  cm., respectively, were used, when the orthodox "descending" (1) or the more recent "ascending" method (14) of development was employed. A volume of the solution to be analyzed, containing about 20  $\gamma$  of each steroid, was applied to a point 6 cm. from one end of the filter paper. It is important that the area to which the solution is applied be not greater than 0.5 cm. in diameter. When the original area was larger than this, the sharpness of the developed chromatogram was impaired. After air drying, the paper was developed at room temperature with water-saturated *n*-butanol in a gas-tight glass chamber, the atmosphere in which was kept saturated with respect both to water and to *n*-butanol. After the moving solvent had run the desired distance (25 to 30 cm.), the paper was removed and the solvent front marked. The paper was dried at room temperature, quickly immersed in the iodoplatinate solution, and then washed in water until only faint background color remained. When the Kraut-Dragendorff reagent was employed, a dilute aqueous solution of hydrochloric acid (about 0.05 *N*) was substituted for the wash water. After treatment with iodoplatinate or iodobismuthate reagent, the positions of the steroid hydrazones were indicated by purple or orange spots, respectively. The iodoplatinate reagent has greater stability, is more sensitive, and produces longer lasting spots. The  $R_F$  of each spot (ratio of the distance moved by the solute to the distance moved by the advancing front of the liquid) was calculated.

### RESULTS AND DISCUSSION

In Table I are listed the ketosteroids studied, grouped according to the number of carbon atoms they contain and in the order of increasing  $R_F$  values. Also tabulated are the numbers of the various polar groups present (see below). The  $R_F$  values listed are averages of six or more runs by the ascending method of development. When run by the descending method, the  $R_F$  values were approximately 10 per cent greater.

Variation in the  $R_F$  value of identical amounts of the same steroid in separate runs was found to be  $\pm 10$  per cent. The factors responsible for this effect have been discussed by Consden *et al.* (1). In addition we found

that, when hydrazones of low mobility were chromatographed, the  $R_F$  values increased slightly as larger amounts of steroid were employed.

TABLE I

$R_F$  Values of Ketosteroid-Reagent T Hydrazones Chromatographed in *n*-Butanol by Ascending Method

Steroids (m.p.)	$R_F$	No. of C atoms	No. of polar groups		
			Carbonyl	Free	Hydroxy
			Reagent T-bound		
Pregnanol-5-trione-3,6,20 (265-267°)*	0.00	21	2	1	1
17-Hydroxyprogesterone (196-203°)	0.06	21	2		1
Pregnanetrione-3,12,20 (199-201°)†	0.10	21	2	1	
Progesterone (125-128°)	0.15	21	2		
$\Delta^4$ -Pregnenetriol-17( $\alpha$ ),20,21-one-3 (223-229°)	0.35	21	1		3
Pregnanediol-3( $\alpha$ ),12( $\alpha$ )-one-20 (170-171°)	0.46	21	1		2
Pregnanedione-3,20 (120-122°)†	0.48	21	1	1	
Allopregnanedione-3,20 (189-193°)†	0.48	21	1	1	
Ethinyltestosterone (280-285°)	0.51	21	1		1
Pregnanol-3( $\alpha$ )-one-20 (140-145°)	0.53	21	1		1
$\Delta^6$ -Pregnenol-3( $\beta$ )-one-20 (184-188°)	0.53	21	1		1
Methyltestosterone (150-158°)	0.50	20	1		1
$\Delta^4$ -Androstenedione-3,17 (170-171°)	0.10	19	2		
Etiocholanedione-3,17 (131-133°)†	0.42	19	1	1	
Androstanedione-3,17 (129-131°)†	0.42	19	1	1	
Androsterone (180-182°)	0.49	19	1		1
Isoandrosterone (176-178°)	0.49	19	1		1
Dehydroisoandrosterone (145-146°)	0.49	19	1		1
Testosterone (154-155°)	0.49	19	1		1
<i>cis</i> -Testosterone (212-215°)	0.49	19	1		1
Etiocholanol-3( $\alpha$ )-one-17 (137-141°)	0.49	19	1		1
Androstanol-17( $\alpha$ )-one-3 (180-182°)†		19		1	1
Androstanone-17 (119-120°)	0.59	19	1		
Androstanone-3 (96-97°)†		19		1	
Estrone (255-263°)	0.47	18	1		1

\* The possibility exists that this compound has split off a molecule of water during formation of the hydrazone to give an  $\alpha,\beta$ -unsaturated 3-keto group. Its behavior, therefore, may be that of a steroid with 3 hydrazide residues of Reagent T.

† The carbonyl at position 3 does not form a hydrazone with Girard's Reagent T (see the text).

In the case of  $\Delta^4$ -androstenedione-3,17, for instance, the  $R_F$  changed from 0.10 with 5  $\gamma$  to 0.13 with 25  $\gamma$ . Similar increases in the amount of tes-

tosterone or other steroids having comparable movement failed to produce significant changes. Because of these many factors influencing the  $R_F$  value, great significance should not be placed upon its absolute magnitude. The more important consideration lies in the relative movement of the various compounds. When chromatographed side by side in equal quantities, this relative movement is constant. The farther a steroid moved in the chromatogram the larger was the spot found. In the case of compounds moving near  $R_F$  0.10, an elliptical spot approximately  $1.5 \times 0.9$  cm. was produced if the initial quantity of steroid was 10  $\gamma$ . With a similar quantity of a compound which moved near  $R_F$  0.50, a spot approximately  $3.0 \times 0.8$  cm. was produced. In all cases the center of the spot was used in calculating  $R_F$ .

The results tabulated may be largely explained by the following assumptions: (1)  $R_F$  is inversely related to water solubility (1). (2) Water solubility is predominately determined by the number of Reagent T hydrazide residues bound to the steroid molecule, and to a lesser degree by the number of other polar groups (uncombined carbonyl and hydroxy groups). (3) Increasing the number of carbon atoms in the molecule decreases its water solubility. With the exceptions designated in Tables I and II, compounds having the same number of carbonyl groups moved, in general, to comparable positions. When, however, one of these was located at position 3 without conjugated unsaturation, the compound either could not be detected (as with androstanone-3 or androstanol-17( $\alpha$ )-one-3) or behaved like a compound having one less ketone group. Thus androstanedione-3,17, eticholanedione-3,17, pregnanedione-3,20, and allopregnanedione-3,20 moved comparably to monoketonic compounds. The polar effect of the "uncombined" carbonyl group in these cases was reflected by the difference in  $R_F$  of androstanone-17 and androstanedione-3,17. It is felt that 3-keto groups, without conjugated unsaturation, do not form hydrazones with Girard's Reagent T sufficiently stable to escape hydrolysis during development of the chromatogram.<sup>3</sup> The effect of the free hydroxy groups was somewhat less marked. This was shown by differences in  $R_F$  between progesterone and 17-hydroxyprogesterone and among the monoketonic pregnanes and pregnenes having 1, 2, and 3 hydroxy groups. The relatively minor influence of non-polar groups in augmenting movement is seen in a comparison of estrone, testosterone, methyltestosterone, and ethinyltestosterone. In the same way, acetylation of hydroxy groups decreased water solubility and increased  $R_F$ ; the  $R_F$  of pregnanediol-3( $\alpha$ ),12( $\alpha$ )-one-20 was 0.46, while that of its diacetate was 0.55.

It is obvious that isomeric steroids cannot be separated by this method.

<sup>3</sup> Cyclohexanone, a cyclic, saturated ketone, also failed to form a stable hydrazone with Girard's Reagent T.

relative to the number of their carbonyl groups forming stable hydrazones with Girard's Reagent T.

4. The secondary effect of other groups within the molecule is discussed.
5. Limitations and possible applications of the procedure are indicated.

The authors are indebted to Dr. W. H. Strain of the Department of Radiology for many pertinent suggestions during this investigation.

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# THE USE OF CONJUGASE PREPARATIONS IN THE MICROBIOLOGICAL ASSAY OF FOLIC ACID\*

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Taka-diestase (1) and a preparation of hog kidney enzyme (2) have been used routinely to liberate folic acid from its conjugates in the microbiological determination of the vitamin (3-6). However, Olson *et al.* (7) reported recently that taka-diestase and certain proteolytic enzymes are of doubtful value in releasing the vitamin from plant tissues. Hog kidney conjugase also does not completely liberate folic acid in every case with fresh plant materials (8) or plant extracts (2, 9, 10). It has been demonstrated that, with homogenates of rat liver, autolysis at pH 7.0 results in a rapid increase in the folic acid content (11-13), whereas at pH 4.5 neither autolysis of the liver nor digestion of heated samples with hog kidney conjugase causes release of the vitamin (13). Apparently there are bound forms of folic acid not hydrolyzable by the conjugase preparations now available. According to Luckey *et al.* (3) no one method could be prescribed to attain maximum folic acid values in all types of materials. Charkey *et al.* (14) also suggest that there may be more than one form of the conjugate present in yeast.

In spite of the wide-spread occurrence in tissues and organs of enzymes capable of converting the conjugated pteroylglutamic acid to the free acid (15), there is little information as to whether conjugases differ in respect to their mechanism of action. In this communication are reported the results of certain preliminary observations which suggest that conjugases may vary in their ability to liberate folic acid or folic acid-active substances from natural sources.

## EXPERIMENTAL

*Methods*—The hog kidney enzyme used in these studies was a clarified water extract of hog kidney prepared and stored frozen as described by

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from Swift and Company, Chicago, Illinois.

We wish to thank the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for synthetic pteroylglutamic acid and pteroyltriglutamic acid and Parke, Davis and Company, Detroit, Michigan, for crystalline vitamin B<sub>9</sub> conjugate used in these studies.

Bird *et al.* (2). The chicken pancreas conjugase (15, 16) was prepared from fresh, frozen chicken pancreas. The pancreas was homogenized with 0.1 M phosphate buffer at pH 7.0 (2 ml. of buffer per gm. of pancreas) in a Waring blender. The homogenate was allowed to autolyze 24 hours at 37° and was then centrifuged to remove fat. The extract was transferred to tubes and frozen before storage. Homogenates of rat liver were prepared by the procedure outlined by Olson *et al.* (13) and were used at a dilution of 1:5. Folic acid activity was determined by using *Streptococcus faecalis* and the turbidimetric method of Luckey *et al.* (17) (the medium being modified by the addition of Salts B (18)). Synthetic pteroylglutamic acid (Lederle) was used for the standard.

*Liberation of Folic Acid from Yeast Samples with Chick Pancreas and Hog Kidney Enzymes*—For hydrolysis with hog kidney enzyme, 5 ml. of a

TABLE I  
*Folic Acid Content of Yeast Samples*

Enzyme used	Sample I. Difco yeast extract	Sample II. Brewers' yeast, dried, non-debit- tered	Sample III. Brewers' yeast, dried, debittered	Sample IV. Dried yeast
	$\gamma$ per gm.	$\gamma$ per gm.	$\gamma$ per gm.	$\gamma$ per gm.
None	3.8	2.2	1.3	2.0
Hog kidney enzyme	30.0	13.2	13.2	15.3
Chick pancreas enzyme	45.0	9.0	9.0	15.0
“ “ “ followed by hog kidney enzyme	125.0	19.0	22.0	16.0

solution or fine suspension, equivalent to 1 to 10 mg. of the air-dry product, were added to 5 ml. of McIlvaine's disodium phosphate-citric acid buffer at pH 4.5 containing 2 ml. of the enzyme preparation. The mixture was incubated under toluene at 37° overnight (18 to 20 hours), heated in a boiling water bath for 2 minutes, cooled, neutralized to pH 6.8 to 7.0, made to a convenient volume, and filtered. Aliquots of the solution were used for microbiological assay. The procedure for hydrolysis with the chick pancreas conjugase was similar. In this case, however, the sample was incubated with 1 ml. of the enzyme preparation in buffer at pH 7.0 for 6 to 8 hours. When the two enzymes were used successively, the mixture, after incubation with the first enzyme, was adjusted to the pH optimum for the second enzyme before its addition. Blanks for the enzyme preparation were subtracted from the values reported. Table I gives a typical set of results obtained.

It may be observed that, with the exception of Sample IV, successive hydrolyses with the two enzymes gave much greater folic acid activity

than the employment of either enzyme alone. The same results were obtained regardless of which enzyme was used first. It appeared as if the two enzymes acted specifically on different types of substrates, although it was quite possible that there was some overlapping in the action of the two enzymes.

With Difco yeast extract as substrate and the two enzyme preparations as above, the values obtained for folic acid by means of *Lactobacillus casei* and the titrimetric method of Teply and Elvehjem (18) were as follows: free folic acid, 3.5  $\gamma$  per gm.; hydrolyzable by hog kidney enzyme, 20.0  $\gamma$  per gm.; hydrolyzable by chick pancreas enzyme, 35.0  $\gamma$  per gm.; and folic acid hydrolyzable by both enzymes, 93.0  $\gamma$  per gm. While the general nature of the results obtained was the same as with *Streptococcus faecalis*, the values were somewhat lower than those obtained with the latter organism (Table I). Higher values with *S. faecalis* have also been reported by Fager *et al.* (8) in various vegetable and plant extracts. Differences in the response of the two microorganisms in the folic acid assay have been recognized (7) and may arise as a result of variability in their utilization of the different forms of folic acid, pteric acid, or the SLR<sup>1</sup> factor (19). Further studies were confined only to the use of *S. faecalis* as the test organism for microbiological assay.

*Liberation of Folic Acid from Yeast by Rat Liver Enzymes*—Since previous workers indicated the existence of two enzyme systems in rat liver, acting at pH 4.5 and 7.0 respectively (13), trials were carried out with 1 ml. lots of rat liver homogenate as the enzyme source and Difco yeast extract (5 ml. of solution containing 1 mg. of yeast extract) as substrate. The incubation time was 4 hours in every case; this period was sufficient for maximum release of folic acid. In a typical experiment, the results obtained with the liver enzymes at pH 4.5, pH 7.0, and at the two pH values successively were 40  $\gamma$ , 50  $\gamma$  and 100  $\gamma$  of folic acid respectively per gm. of yeast extract after allowing for the blanks obtained by incubating the liver preparations alone. The enzyme systems in rat liver presumably correspond to both the hog kidney and chick pancreas enzymes.

*Liberation of Folic Acid from Rat Liver Homogenate by Hog Kidney and Chick Pancreas Enzymes*—In the following experiments, 1.0 ml. of heated rat liver homogenate, containing 0.2 gm. of liver, was used as substrate. The values obtained by action of the rat liver enzymes are included for comparison (Table II).

The observations of Olson *et al.* (13) that maximum liberation of folic acid from liver takes place only at pH 7.0 are borne out by these data (Table II); at this pH, chick pancreas enzyme releases nearly as much folic acid as the liver enzyme itself. Further, the inability of hog kidney

<sup>1</sup> *Streptococcus lactis* R. This organism is also known as *Streptococcus faecalis* R.

enzyme to free the vitamin from its combination in the liver corresponds to that of the liver enzyme at pH 4.5. The values (Sample I) obtained after autolysis at pH 4.5 or treatment with hog kidney enzyme at this pH were somewhat higher and less consistent than when the samples were collected under more rigid conditions of refrigeration (Sample II). Apparently the liver enzymes release folic acid rapidly in the intact liver at room temperature.

TABLE II  
*Release of Folic Acid from Rat Liver by Conjugases*

Treatment	Folic acid values, γ per gm. liver	
	Sample I	Sample II
Heated liver, unincubated	1.0	0.46
“ “ + hog kidney enzyme, pH 4.5, overnight	1.3	0.43
“ “ + chick pancreas enzyme, pH 7.0, 8 hrs.	2.3	
“ “ + fresh liver, pH 7.0, 4 hrs.	2.5	
Fresh liver, autolyzed at pH 7.0, 4 hrs.	2.5	2.5
“ “ “ “ 4.5, 4 “	1.6	0.50
“ “ “ “ 4.5 for 4 hrs. and at pH 7.0 for 4 hrs.	2.3	

TABLE III  
*Release of Folic Acid on Incubation of Enzyme Mixtures*

	Folic acid values					
	Sample I	Sample II	Sample III	Sample IV	Sample V	Sample VI
	γ	γ	γ	γ	γ	γ
2 ml. hog kidney enzyme, pH 4.5, overnight	0.02	0.01	0.01	0.03	0.01	0.01
1 ml. chick pancreas enzyme, pH 7.0, 8 hrs.	0.48	0.65	0.35	0.50	0.45	0.14
2 ml. hog kidney enzyme + 1 ml. chick pancreas enzyme, pH 4.5, overnight	0.65	0.92	0.40	0.61	0.50	0.22

Since the liver conjugate is largely hydrolyzable at pH 7.0, it would probably appear that this is the form in which folic acid reserve is held in the liver. With yeast, evidently there is more than one form of substrate.

*Liberation of Folic Acid from Hog Kidney and Chick Pancreas Enzyme Mixtures*—In the course of the foregoing experiments with mixtures of hog kidney and chick pancreas enzymes, it was noticed that, in the blanks obtained for the enzymes alone, the folic acid amounted frequently to much more than the sum of the values for the individual enzymes. Some of the



results obtained with different lots of the two enzyme preparations are given in Table III.

In these experiments, the mixture of enzymes was in each case incubated overnight at pH 4.5 before microbiological assay, since this was the procedure followed whenever the enzymes were used successively with the various natural sources of folic acid compounds studied. Chick pancreas enzyme, in addition to giving a high blank for folic acid, obviously contains a folic acid compound hydrolyzable by hog kidney enzyme at pH 4.5, thus accounting for the increased folic acid in the mixture of enzymes. It was ascertained in one instance (Sample I, Table III) that, when the mixture of enzymes was incubated at pH 7.0 for 8 hours so as to correspond to the addition of enzymes for successive hydrolyses by hog kidney enzyme

TABLE IV  
*Folic Acid Content of Some Natural Materials*

	Untreated	With chick pancreas enzyme	With hog kidney enzyme	With both enzymes
	$\gamma$ per gm.	$\gamma$ per gm.	$\gamma$ per gm.	$\gamma$ per gm.
Dry liver preparation, unknown origin	0.7	1.5	1.4	3.0
Condensed fish solubles, 50% total solids	0.04	0.20	0.12	0.30
Dried egg yolk	0.11	0.52	0.47	0.55
Whole " I, fresh basis		0.020	0.033	0.030
" " II, " "		0.063	0.065	0.060
Spinach, dried	2.42	6.15	7.05	9.25
Mustard greens, dried	0.97	4.55	4.10	4.75
Soya flour	0.46	2.55	2.80	3.35

followed by chick pancreas enzyme, the folic acid content at the end of treatment was only slightly higher than the sum of the values for the individual enzymes (actually, 0.5  $\gamma$ ). Hog kidney enzyme preparations, in addition to giving a low blank, do not presumably contain other forms of folic acid hydrolyzable at pH 7.0.

*Enzymatic Liberation of Folic Acid from Other Natural Sources*—The values obtained, by the procedure outlined above, for a few other materials are listed in Table IV. In the case of dried liver and condensed fish solubles, higher values for folic acid were secured by successive hydrolyses. The results with egg samples and with dried greens would suggest that in these cases folic acid, or at any rate a large part of it, is present in a form which is mostly released by hog kidney enzyme alone and perhaps also by chick pancreas enzyme.

*Liberation of Folic Acid from Pteroyltriglutamic Acid and Pteroylhepta-*

*glutamic Acid*—With a view to gaining an insight into the mechanism of action of the two enzymes, crystalline pteroylheptaglutamic acid (Parke, Davis) and synthetic pteroyltriglutamic acid (Lederle) were used as substrates for hydrolysis. With the use of 5 ml. lots of solutions of the two conjugates (1 ml. = 0.1  $\gamma$  of folic acid) the percentage recoveries of folic acid are given in Table V.

While pteroyltriglutamic acid is completely hydrolyzed by both chick pancreas and hog kidney enzymes, it may be seen that the heptaglutamic acid is not fully hydrolyzed by either enzyme, the hog kidney enzyme being, however, more powerful of the two for this conjugate. The only available supply of pteroylheptaglutamic acid was a 10  $\gamma$  per ml. solution and it is possible that this solution had undergone some change or degradation into other forms not hydrolyzable by hog kidney enzyme. From a qualitative point of view, however, these results again emphasize the nature of the

TABLE V

*Release of Folic Acid from Pteroyltriglutamic Acid and Pteroylheptaglutamic Acid*

	Per cent folic acid recovered			
	Without hydrolysis	With chick pancreas enzyme	With hog kidney enzyme	With both enzymes
Pteroyl triglutamate.....	4.7	94	100	100
Pteroyl heptaglutamate 1.....	6.0	20	76	105
“ “ 2.....	6.7	36	78	92

differences in the mode of action of the two enzyme preparations. The hydrolytic action of chick pancreas enzyme was extended for 24 hours, but there was no significant difference in the extent of release of available folic acid, thus showing definitely that folic acid is not fully liberated from heptaglutamic acid by this enzyme.

#### DISCUSSION

Luckey *et al.* (3) had observed that, when various hydrolytic methods, enzymatic as well as non-enzymatic, were compared, certain treatments gave higher values than others for some samples, while the reverse was true for others. The values obtained for yeast extract (Difco) by various treatments were so variable that they concluded it was difficult to determine its folic acid content. Bird *et al.* (2) obtained low results by microbiological assay for certain plant extracts as compared to chick assay and suggested that conjugase inhibitors might account for this observation. However, the fact that different treatments are required to give maximum values

for different materials would indicate that the compounds in the folic acid group are bound in natural materials by different chemical unions.

On the basis of the results presented here, it is not possible to outline a definite procedure for the use of conjugase preparations which would result in the maximum liberation of folic acid in all cases. However, it seems definite that conjugases differ in regard to their specificity of hydrolytic action on folic acid-active materials, although a certain amount of overlapping may occur. The suggestion of Olson *et al.* (7) that certain folic acid complexes are first degraded to pteroylheptaglutamic acid and subsequently to pteroylglutamic acid could account for the observation reported here that successive hydrolytic action by more than one enzyme does not necessarily result in additive values for folic acid. Only when the chemical nature of the conjugates or other folic acid complexes is known will it be possible to ascertain whether conjugases, so widely distributed in nature, vary in their mechanism of action. Meanwhile it should be possible to standardize the conditions of time, temperature, and pH in the acidic hydrolysis of materials, first reported by Briggs *et al.* (20, 21) and later by others (22, 3) for the liberation of free folic acid from its bound forms.

#### SUMMARY

1. The use of hog kidney and chick pancreas enzymes for the release of folic acid from yeast samples resulted in different values for the vitamin as assayed microbiologically, with use of either *Streptococcus faecalis* and the turbidimetric method or *Lactobacillus casei* and the acidimetric method. Successive hydrolyses by the two enzymes gave much higher values for folic acid than when they were used singly.

2. Similar differences were observed in the enzymatic hydrolysis of yeast extract by the rat liver enzymes at pH 4.5 and 7.0, maximum liberation of folic acid being obtained by the use of the two enzymes successively.

3. Rat liver homogenate increased in folic acid content on autolysis at pH 7.0 or when hydrolyzed by chick pancreas enzyme, but not on autolysis at pH 4.5 or when hydrolyzed by hog kidney enzymes.

4. It is shown, from data on the enzymatic treatment of a few other natural materials and of pteroyltri- and pteroylheptaglutamic acids, that conjugases may vary in their ability to hydrolyze different forms of folic acid that may occur in plant materials and that therefore their use does not necessarily result in maximum folic acid values.

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# THE PROBABLE EXISTENCE OF A COENZYME FORM OF BIOTIN

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(Received for publication, August 20, 1948)

It has been demonstrated that biotin is concerned with the enzymatic decarboxylation of oxalacetate (1-4), the reversible deamination of aspartate (5, 6), and the deamination of serine and threonine (5, 6). By analogy with other vitamins it might be suspected that biotin does not function in these reactions as such, but is converted into a coenzyme form. The evidence outlined below suggests that a coenzyme form of biotin does exist in certain natural materials.

## Procedures and Results

First, as shown in Table I,  $10^{-4}$   $\gamma$  of biotin was required to stimulate the aspartic acid deaminase of aged cell suspensions of *Proteus vulgaris* rendered deficient by the techniques previously employed (1, 5, 6). 1  $\gamma$  of yeast extract (Difco) had equivalent activity. Microbiological assay of untreated yeast extract for biotin with *Saccharomyces cerevisiae* (7) revealed that 1  $\gamma$  of yeast contained only  $10^{-6}$   $\gamma$  of biotin. Therefore, the yeast extract was approximately 100-fold more active than could be accounted for by its assayable biotin content.

Second, by grinding vacuum-dried preparations of cells of *Escherichia coli* (Gratia strain) it has been possible to obtain a cell-free preparation of aspartic deaminase in a partially resolved state. These preparations are not stimulated (as shown in Table II) by biotin alone, but can be activated by biotin and adenylic acid (adenosine-5-phosphate), and by yeast extract. In Preparations A and B, the biotin plus adenylic acid was initially as effective as yeast extract, but after refrigeration for 24 hours the preparations were activated only by the yeast extract. The activity of the yeast is not therefore attributable to its adenylic acid content. The data can be interpreted to mean that during the refrigeration the enzyme system utilizing biotin and adenylic acid for activation has been destroyed, while the yeast extract maintains its activity because it contains the preformed coenzyme. In Preparations C and D the grinding alone has destroyed the ability of biotin and adenylic acid to activate the system but yeast extract remains effective.

The following procedure resulted in complete destruction of the stimulatory material. The yeast extract was ashed by incineration, boiled with

TABLE I

*Stimulation of Aspartic Acid Deaminase in Proteus vulgaris by Biotin and Yeast Extract*

Biotin per 2 ml.	Ammonia nitrogen produced	Yeast extract		Ammonia nitrogen produced
		Per 2 ml.	Biotin* equivalent	
$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$
0	3.8	0		3.8
1	8.6			
$10^{-1}$	10.5			
$10^{-2}$	10.9	1000	$10^{-2}$	9.5
$10^{-4}$	10.5	100	$10^{-4}$	9.2
$10^{-5}$	4.8	10	$10^{-5}$	9.6
$10^{-6}$	4.2	1	$10^{-6}$	9.6
		0.1	$10^{-7}$	4.1

Cells of *Proteus vulgaris* grown for 16 hours in media containing 1 per cent each of yeast extract and tryptone, 0.5 per cent phosphate, and 0.1 per cent formate. Harvested cells aged in M phosphate, pH 4, at 26° for 30 minutes. Reaction run at 37°, pH 4, in 0.5 M phosphate for 30 minutes.

We are grateful to Merck and Company, Inc., for generous supplies of biotin.

\* Determined by *Saccharomyces cerevisiae* assay, 1  $\gamma$  =  $10^{-6}$   $\gamma$  of biotin.

TABLE II

*Aspartic Acid Deamination in Cell-Free Preparations*

Preparation	Treatment	Ammonia nitrogen produced				
		No. addition	Biotin, 0.05 $\gamma$ per ml.	Adenylic acid, 50 $\gamma$ per ml.	Biotin + adenylic acid	Yeast extract* 0.5 mg. per ml.
		$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$
A	None	2.4	2.6	2.1	4.5	5.5
"	Refrigerated 24 hrs.	2.1	2.1	2.2	2.0	6.7
B	None	2.1			7.9	7.9
"	Refrigerated 24 hrs.	2.0			2.5	6.0
C	None	3.4	3.8	3.2	4.0	7.0
D	"	3.6	4.0	3.4	4.2	8.9

Cells of *Escherichia coli* (Gratia) grown 17 hours at 27° in media containing 1 per cent each of yeast extract and pepticase, 0.5 per cent phosphate, and 0.6 per cent glucose. Cells harvested by centrifugation, washed once with water, and dried *in vacuo* over Drierite. Dried cells ground in a ball mill *in vacuo* 12 hours, suspended in water, centrifuged, and clear supernatant used as cell-free juice. Reaction run at 37°, pH 7, in 0.5 M phosphate for 60 minutes; cell-free juice per tube equivalent to 20 mg. of dried cells; stopped with trichloroacetic acid, centrifuged, and an aliquot of the supernatant analyzed for ammonia by nesslerization with a Klett-Summerson photoelectric colorimeter. The increase in ammonia over the samples incubated for 60 minutes without aspartic acid was taken as an index of deamination.

We are grateful to the Ernst Biscoff Company for supplies of adenylic acid.

\* Equivalent to 0.005  $\gamma$  of biotin by assay with *Saccharomyces cerevisiae*.

concentrated nitric acid, and with concentrated perchloric acid. This material was then suspended in water, neutralized with sodium hydroxide, and diluted to 1 per cent with respect to yeast extract. This solution was completely inactive in the stimulation of aspartic acid deaminase in aged cell suspensions of *Proteus vulgaris*, and suggests that the stimulatory material is organic in nature.

#### SUMMARY

These results suggest that a coenzyme form of biotin does exist, that it is either more active than biotin or not assayable by *Saccharomyces cerevisiae*, and that adenylic acid is somehow concerned with its formation.

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# AN IMPROVED ASSAY FOR A GROWTH FACTOR IN LIVER EXTRACTS\*

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(Received for publication, August 23, 1948)

Previous work (1) has shown that liver contains a factor which produces a growth response in rats receiving a ration containing all the known vitamins. Bosshart *et al.* (2) have also described a growth-stimulating effect of liver in rats fed an otherwise complete diet. Jaffé and Elvehjem (3) and later Sporn *et al.* (4) described assay procedures for this factor. They observed a growth response of 5 to 10 gm. per week for periods of 3 to 5 weeks when liver preparations were given, but this response was not sufficient for a completely satisfactory assay.

The present work was undertaken to improve the assay procedure and to study possible relationships between this factor and the antipernicious anemia factor found in liver.

Ershoff (5) and Bethel *et al.* (6) have reported that liver completely counteracts the retardation of growth observed in immature rats when fed a diet high in thyroid. From a study of previous results (3, 4) it was observed that some of the rats on the basal ration grew as well as those on the supplemented ration. These data indicate that some of the rats had sufficient stores of the factor in question to grow normally to maturity, while those giving a response to liver suffered from a border line deficiency. It appeared possible that feeding thyroid or a thyroid-active substance as a supplement to the basal ration might increase the metabolic rate of the rat sufficiently to cause a greater requirement and thereby allow a more rapid depletion of the factor.

## EXPERIMENTAL

Male, weanling rats (Sprague-Dawley) weighing between 40 and 50 gm. were used in all the experiments. They were kept in individual cages and were given *ad libitum* distilled water and the corn-soy bean meal ration

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

We are indebted to Merck and Company, Inc., Rahway, New Jersey, for crystalline vitamins, to Dr. B. L. Hutchings of the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for synthetic folic acid, and to the Abbott Laboratories, Inc., North Chicago, Illinois, for experimental liver extracts.

described earlier (3). The ration has the following composition: whole ground yellow corn 46.35, commercial soy bean meal 46.35, corn oil (Mazola) 5, cystine 0.3,  $\text{CaHPO}_4$  0.92,  $\text{CaCO}_3$  0.6, iodized sodium chloride 0.44, and  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$  0.04 in gm.; thiamine 0.3, riboflavin 0.3, niacin 2, pyridoxine 0.2, pantothenic acid 2, folic acid 0.025, biotin 0.01, inositol 10, choline 100, and *p*-aminobenzoic acid 25 in mg.

Vitamins A and D were administered as oleum percomorphum diluted 1:4 with corn oil and fed at the rate of 2 drops per week. Five rats were used in each group. Typical figures for the average weekly gain in weight

TABLE I  
*Effect of Liver Preparations on Growth of Rats Receiving Basal Ration or Iodinated Casein Ration*

Ration	U. S. P. unit per day	Liver preparation	Average gain per wk.; 4 wk. period
Basal .....			gm.
" .....	1	Reticulogen (Lilly)*	29
" .....	0.5	" "	40
" + iodinated casein.....			40
" + " " .....	1	Reticulogen	21
" + " " .....	0.5	"	37
" + " " .....	0.05	"	41
" + " " .....	1	E-567 (Abbott)†	32
" + " " .....	0.5	E-567 "	38
" + " " .....	1	E-1125 " †	35
" + " " .....	1	Armour†	26
" + " " .....	1	E-1043 (Abbott)†	39
" + " " .....	0.5	E-1043 "	18
			20

\* 20 U. S. P. units per cc.

† 15 U. S. P. units per cc.

of rats on this ration and on the same ration when they received liver extract are given in Table I. When 0.06 per cent iodinated casein<sup>1</sup> was added to the basal ration, a definite decrease in growth rate was observed. If the rats on this ration were given the same liver preparations, the gain in weight was equal to that obtained with the original basal ration. Therefore the addition of iodinated casein allowed a greater difference in growth between the negative and positive controls. Recent work has shown that optimum levels of iodinated casein may vary with the season of the year.

<sup>1</sup> Supplied by Dr. W. R. Graham, Cerophyl Laboratories, Inc., Kansas City, Missouri. (According to their analyses the protamone contained 3.07 per cent thyroxine.)

During the summer months better results may be obtained when 0.1 per cent of iodinated casein is used.

All liver preparations were administered intraperitoneally in amounts equivalent to 1 U. S. P. unit or less per day. The injections were actually made every other day but the levels are expressed as equivalent units per day. It is apparent from the results summarized in Table I that several preparations, including reticulogen (Lilly), an Abbott preparation, and an

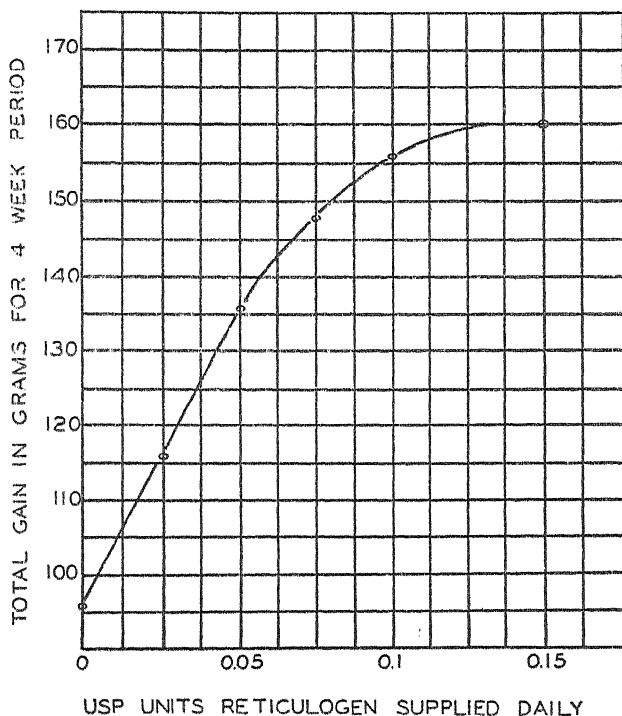


FIG. 1. Growth response in rats given graded doses of reticulogen

Armour preparation, gave maximum growth. However, certain preparations failed to produce a significant response.

In future studies reticulogen (Lilly) was chosen as a standard and graded levels were given to separate groups of rats placed on the iodinated casein ration. The results are presented in Fig. 1. It appears that the best assay range falls between 0.025 and 0.1 U. S. P. unit daily. The level of 0.15 unit gave only a slightly better growth than the 0.1 level and the growth at this level was equal to that obtained earlier with 0.5 unit. Although graded responses were obtained with this procedure, the method was time-

TABLE II  
*Response of Rats to Liver Preparations after 4 Week Depletion Period*

U. S. P. unit	Daily supplement	Average gain 1st wk.
	Preparations used	gm.
	None	17
1	Reticulogen	44
0.5	"	45
0.1	"	45
0.05	"	35
1	E-1125 (Abbott)	15
1	E-1126 (Armour)	48
	Alcohol extract of fish solubles,* 2%	29
	" " " " " 6%	44

\* Added to ration.

TABLE III  
*Comparison of Rat Growth Units in Commercial Liver Preparations and Antipernicious Anemia Units As Shown on Labels*

U. S. P. unit per day	Liver preparation	Average weight gain		Rat units per U. S. P. unit
		1st wk.	Total gain 2 wks.	
		gm.	gm.	
None	None	23	35	
0.1	Reticulogen	42	77	10
0.05	"	33	59	10
0.025	"	29	48	10
0.2	370 (Lilly)	46	82	
0.1	370 "	42	75	
0.05	370 "	43	69	15
0.2	Lederle, refined*	43	80	
0.1	" "	44	78	10
0.05	" "	32	59	10
0.2	Armour*	29	50	1.5
0.1	"	29	43	1.5
0.05	"	20	33	
0.2	2505 (Sharp and Dohme)	30	59	2.5
0.1	2505 " " "	27	53	2.8
0.05	2505 " " "	29	47	

\* 15 U. S. P. units per cc.

consuming, since it was necessary to inject the samples to be tested over a 4 week period. The rats receiving the basal ration continued to show a growth of less than 20 gm. per week during the 5th and 6th weeks on ex-

periment. However, when an active liver preparation was injected during the 5th week a marked growth response was obtained. The values given in Table II show that the same relative activity for different preparations was obtained by this procedure as by the longer method. Data are also included to show that an alcohol extract of condensed fish solubles gave a maximum growth response when fed at a high level.

Therefore, attempts were made to shorten the assay procedure by reducing the time the rats were maintained on the basal ration before supplementation was started. The final procedure involves a 2 week depletion period followed by a 2 week assay period, during which time the sample is administered intraperitoneally on alternate days. When active liver preparations were tested by this procedure the rats that received the supplement during the 2 week period gained approximately twice as rapidly as the unsupplemented animals. 1 rat unit is defined arbitrarily as the quantity of the factor necessary daily to produce a growth response of  $75 \pm 5$  gm. during the 2 week assay period. This rate of growth is approximately optimum and is obtained with 0.1 U. S. P. unit of reticulogen used.

In Table III results are presented for assays carried out on several commercial liver extracts. In each case the activity obtained in the rat assay is compared with the pernicious anemia potency indicated on the label.

#### DISCUSSION

A significant growth response has been obtained in rats fed a diet consisting mainly of yellow corn and soy bean meal together with iodinated casein and the available vitamins when commercial pernicious anemia liver extracts are administered. Quantitative responses can be obtained when liver preparations are injected on alternate days during a 1 or 2 week period after the rats have received the basal ration for 2 weeks. The factor which is measured in this assay is probably the same as that studied by Bethel *et al.* (6), although lower levels of thyroid-active material have been used in the present study. It also appears that the factor is the same as that described by Robblee *et al.* (7) for the chick. Recently these workers (8) have shown that the requirement of a factor in liver and condensed fish solubles is higher when the basal ration contains iodinated casein. Some of the same preparations have been tested in the chick and in the rat and comparable activities have been observed.

Preliminary fractionation studies have shown that the factor was not extracted from an aqueous solution by ether and only to a slight extent by butanol. It was precipitated by lead, barium, silver, and mercury salts and by phosphotungstic acid. At pH 3 it was adsorbed on fullers' earth but only to a slight extent by Super Filtrol. Although we have not had an opportunity to test vitamin B<sub>12</sub> (9), there is no evidence available to

indicate that vitamin B<sub>12</sub> would not give a response. When the rat growth activity of a number of commercial liver preparations was compared with the antipernicious anemia activity, as indicated on the label, good correlation was observed in at least three preparations. Wider variations were observed in the other cases but even here the variations were not greater than 10-fold. When one realizes the difficulties encountered in the human assays, it is not surprising that these variations are encountered.

#### SUMMARY

An improved rat assay has been developed for a growth factor in liver extracts. This method is satisfactory for the quantitative estimation of this factor in commercial liver preparations.

Assays have been made on a number of liver preparations and the results compared with the U. S. P. antipernicious anemia potency given on the label.

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# GLUTAMIC ACID ANTIMETABOLITES: SULFOXIDES AND SULFONES DERIVED FROM METHIONINE HOMOLOGUES; $\beta$ -HYDROXY-GLUTAMIC ACID\*

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(Received for publication, August 24, 1948)

In previous studies, it was shown that the sulfoxide derived from methionine (MSO) is an effective antimetabolite against glutamic acid in the metabolism of *Lactobacillus arabinosus*, probably by blocking the amidation of the amino acid to glutamine (2, 3). The sulfoxide derived from benzylhomocysteine is also an effective antimetabolite against glutamic acid, whereas the sulfoxide derived from ethionine is ineffective in comparable concentrations (3). It appeared of interest to study the effect on antimetabolite activity of increasing the chain length of the S-alkyl substituents. The sulfoxides and sulfones derived from *n*-propyl-, *n*-butyl-, *n*-amyl-, and *n*-hexylhomocysteine were therefore prepared and tested.

A study of the effect of  $\alpha$ -aminopimelic acid and of  $\beta$ -hydroxyglutamic acid as antimetabolites is also included in this report.

## EXPERIMENTAL

*S-Alkylhomocysteines*—S-*n*-Propyl-, S-*n*-butyl-, S-*n*-amyl-, S-*n*-hexyl-, and S-*n*-laurylhomocysteine were prepared from S-benzyl-DL-homocysteine and the appropriate alkyl bromide according to the method of Dyer for the preparation of ethionine (4) (see Table I). The yields were over 90 per cent of the theoretical. Isopropyl bromide, tertiary butyl bromide, and tertiary amyl bromide did not condense with the sodium mercaptide of homocysteine in liquid ammonia.

*Sulfoxides and Sulfones Derived from S-Alkylhomocysteines*—The sulfoxides were prepared by a slight modification of the method of Toennies and Kolb (5, 3) and the sulfones by a method of the same authors (6). (See Table II.) The sulfoxides and sulfones derived from the S-*n*-laurylhomocysteine could not be obtained in analytically pure form.

DL- $\alpha$ -Aminopimelic acid has been previously prepared from the corre-

\* Supported by grants from the Rockefeller Foundation, the New York Foundation, and the Williams-Waterman Fund of the Research Corporation. A preliminary report has appeared elsewhere (1).

sponding oxime (7). We prepared it by treating the condensation product of phthalimidomalononic ester and tetramethylene bromide with potassium cyanide and subsequently hydrolyzing the resulting nitrile.

0.2 mole of sodium phthalimidomalononic ester was heated with 1 mole of tetramethylene bromide at 160–165° for 3 hours, then at 175–185° for 9 hours. When cool, the mixture was filtered and the sodium bromide was washed with a small amount of alcohol. The combined filtrates were

TABLE I  
*S-Alkylhomocysteines*

	Melting point, uncorrected	N, calcu- lated	N, found
	°C.	per cent	per cent
<i>S-n</i> -Propyl-DL-homocysteine, $C_7H_{15}SO_2N$ (177.2)	249	7.9	7.9
<i>S-n</i> -Butyl-DL-homocysteine, $C_8H_{17}SO_2N$ (191.3)	254	7.3	7.2
<i>S-n</i> -Amyl-DL-homocysteine, $C_9H_{19}SO_2N$ (205.3)	250–252	6.8	7.0
<i>S-n</i> -Hexyl-DL-homocysteine, $C_{10}H_{21}SO_2N$ (219.3)	245–248	6.4	6.4
<i>S-n</i> -Lauryl-DL-homocysteine, $C_{16}H_{33}SO_2N$ (303.4)	212–215	4.6	4.5

TABLE II  
*Sulfoxides and Sulfones Derived from S-Alkylhomocysteines*

Acid		Melting point, uncorrected	N, calcu- lated	N, found
		°C.	per cent	per cent
<i>γ-n</i> -Propylsulfinyl-DL- $\alpha$ -amino- butyric	$C_7H_{15}SO_3N$ (193.2)	230	7.2	7.1
<i>γ-n</i> -Butylsulfinyl-DL- $\alpha$ -aminobutyric	$C_8H_{17}SO_3N$ (207.3)	238	6.7	6.6
<i>γ-n</i> -Amylsulfinyl-DL- $\alpha$ -aminobutyric	$C_9H_{19}SO_3N$ (221.3)	235–238	6.3	6.3
<i>γ-n</i> -Hexylsulfinyl-DL- $\alpha$ -aminobutyric	$C_{10}H_{21}SO_3N$ (235.3)	232–235	6.0	5.8
<i>γ-n</i> -Propylsulfonyl-DL- $\alpha$ -aminobutyric	$C_7H_{15}SO_4N$ (209.2)	240	6.7	6.6
<i>γ-n</i> -Butylsulfonyl-DL- $\alpha$ -aminobutyric	$C_8H_{17}SO_4N$ (223.2)	251	6.3	6.1
<i>γ-n</i> -Amylsulfonyl-DL- $\alpha$ -aminobutyric	$C_9H_{19}SO_4N$ (237.3)	242–245	5.9	5.8
<i>γ-n</i> -Hexylsulfonyl-DL- $\alpha$ -aminobutyric	$C_{10}H_{21}SO_4N$ (251.3)	245–248	5.6	5.4

evaporated to dryness *in vacuo*. The residue was dissolved in 200 ml. of ethanol, 40 gm. of KCN were added, and the mixture was heated under a reflux with stirring for 20 hours. When cool, the mixture was filtered, the precipitate washed with ethanol, and the combined filtrates evaporated to a thick syrup *in vacuo*. The syrup was dissolved in 60 ml. of 95 per cent ethanol, 120 ml. of 5 N NaOH were added, and the solution was heated on the steam bath for 5 hours until no more ammonia was evolved. The solution was cooled to 0° and acidified with 6 N HCl to Congo red. After



standing in the ice box for 12 hours, 18.5 gm. of crystalline product were obtained. The crystals were hydrolyzed in 100 ml. of 6 N HCl under a reflux for 5 hours. After being cooled, the phthalic acid was filtered and the filtrate was evaporated to dryness *in vacuo*. By repeated addition and evaporation of water *in vacuo* the excess HCl was removed. The residue was dissolved in 60 ml. of hot water and neutralized with 6 N ammonia until the solution was just alkaline to Congo paper. After 12 hours in the ice box, the crystals of  $\alpha$ -aminopimelic acid were filtered off. An additional crop was collected after concentration of the mother liquor. The pooled crystals were recrystallized three times from the minimal amount of hot water in the presence of a small amount of charcoal in the first recrystallization: 4.2 gm. of white crystals were obtained with a melting point of 219–220° (corrected), which could not be raised by further recrystallization (225° (7)). The over-all yield, based on the amount of phthalimidomalonic acid used, was 12 per cent.

$C_7H_{13}NO_4$ . Calculated, C 48.0, H 7.5, N 8.0; found, C 47.9, H 7.4, N 8.0

Synthetic  $\beta$ -hydroxyglutamic acid was kindly supplied by Dr. H. E. Carter and Dr. O. Touster of the University of Illinois. It was an unresolved mixture of the four isomers.

The bacteriological assay was carried out as described previously (3) with the medium of Hac, Snell, and Williams (8) and turbidimetric measurement of bacterial growth. The compounds were dissolved in a previously autoclaved culture medium at pH 6.3 and were then sterilized by filtration. The lauryl and hexyl derivatives as well as the S-amyl sulfone were too insoluble for testing. All comparisons were made at the same glutamic acid or glutamine concentration,  $0.21 \times 10^{-3}$  M.

#### RESULTS AND DISCUSSION

The maximal inhibition produced by each compound is reported in Table III. The sulfoxide and the sulfone of ethylhomocysteine were the weakest inhibitors in each series. With increasing alkyl chain length, however, growth inhibition tends to become more pronounced with both the sulfoxides and the sulfones, but none of them approximated MSO as an antimetabolite. The inhibition by these homologues, unlike that of MSO, is not completely reversed by glutamine (Table IV); apparently they affect mechanisms other than amidation as well.

The titration curves of the sulfoxides and sulfones derived from methionine and ethionine were obtained the usual way. Since the titration curves of the derivatives of methionine and ethionine coincided, it appears that the different antimetabolite potency is dependent on some inherent

property of the structure other than the dissociation of the functional groups.

$\alpha$ -Aminopimelic acid, like  $\alpha$ -aminoadipic acid (3), was found not to inhibit bacterial growth in concentrations up to 0.045 M.

TABLE III

*Relative Inhibitory Activity of Sulfoxides and Sulfones Derived from Methionine Homologues*

L-Glutamic acid,  $0.21 \text{ M} \times 10^{-3}$ .

Sulfoxides derived from	$\text{M} \times 10^{-3}$	Molar ratio, inhibitor-glutamic acid	Inhibition  per cent
Methionine.....	7.3	35:1	88
".....	9.7	46:1	100
Ethionine.....	56	260:1	52
S-Propylhomocysteine.....	52	247:1	83
S-Butylhomocysteine.....	38.6	184:1	56
S-Amylhomocysteine.....	36	172:1	56
Sulfones derived from			
Methionine.....	17.7	84:1	72
Ethionine.....	32.8	156:1	72
S-Propylhomocysteine.....	30.6	146:1	72
S-Butylhomocysteine.....	18	85:1	53

TABLE IV

*Effect of Glutamine on Growth Inhibition Produced by Sulfoxides and Sulfones*

Glutamic acid $\text{M} \times 10^{-3}$	Glutamine $\text{M} \times 10^{-3}$	Sulfoxides derived from	$\text{M} \times 10^{-3}$	Inhibition  per cent
0.21	0	Methionine	9.7	100
0	0.21	"	9.7	5
0.21	0	S-n-Amylhomocysteine	36.0	56
0	0.21	"	36.0	20
Sulfone derived from				
0.21	0	S-n-Propylhomocysteine	30.6	72
0	0.21	"	30.6	30

$\beta$ -Hydroxyglutamic acid inhibited growth completely in a concentration of 0.025 M when the concentration of glutamic acid was 0.00082 M. The inhibition was largely overcome either by a 3-fold increase of glutamic acid or by a small amount of glutamine (Table V).

This result indicates that  $\beta$ -hydroxyglutamic acid acts by the same mechanism as does MSO; *i.e.*, by an interference with amidation. The two compounds resemble each other also in the degree of activity; the antibacterial index of the hydroxyglutamic acid was 30 at the glutamic acid concentrations tested, as compared with 30 to 50 for MSO (3). They differed in one respect: the inhibition by  $\beta$ -hydroxyglutamic acid was not overcome by prolonged incubation. But this difference is more apparent than real. The sulfoxide is reduced to methionine during prolonged bacterial metabolism, and thus loses its antimetabolite activity. Evidently

TABLE V  
 *$\beta$ -Hydroxyglutamic Acid As Antimetabolite, 20 Hour Incubation*

L-Glutamic acid	Glutamine	$\beta$ -Hydroxyglutamic acid	Growth (optical density)	Inhibition
$M \times 10^{-3}$	$M \times 10^{-3}$	$M \times 10^{-3}$		per cent
0.82			0.36	0
0.82		25	0.02	95
2.4		25	0.30	18
0.55	0.27	25	0.35	3

*$\beta$ -Hydroxyglutamic Acid As Substitute for Glutamic Acid, 72 Hour Incubation*

L-Glutamic acid	$\beta$ -Hydroxyglutamic acid	Growth (optical density)
$M \times 10^{-3}$	$M \times 10^{-3}$	
0	0	0
0.82	0	0.47
0	0.74	0
0	1.48	0
0.41	0	0.41
0.41	0.37	0.39

the bacteria are unable to alter  $\beta$ -hydroxyglutamic acid. Further evidence for this assumption is the inability of the bacteria to use  $\beta$ -hydroxyglutamic acid as a nutrient either in the complete absence of or in the presence of suboptimal amounts of glutamic acid (Table V).

Since the hydroxyglutamic acid used was a mixture of four isomers, it is likely that one of the isomers accounted for most of the activity, as in the case of the sulfoxide derived from methionine (9).

The mechanism of inhibition by hydroxyaspartic acid in *Escherichia coli* appears to be different, for asparagine is no more effective in counteracting that inhibition than aspartic acid (10). Both MSO and hydroxyglutamic acid appear to be competitive inhibitors acting on the enzyme system,

which converts glutamic acid to its amide.<sup>1</sup> Studies of antimetabolites on intact organisms cannot determine whether the antimetabolite acts by interference with an enzyme reaction within the cell or by blocking the penetration of the metabolite. Recent observations on the isolated enzyme system responsible for the amidation of glutamic acid have shown that MSO is a true competitive inhibitor (12).

Although our studies indicate that glutamine synthesis is an obligatory step in the metabolism of certain bacteria, it has recently been shown with the aid of alkyl-substituted amides of glutamic acid, which apparently block the deamidation of the amide, that free glutamic acid is also necessary for bacterial metabolism (13).

This is not unexpected, since glutamic acid and glutamine apparently have different functions in metabolism. The amino acid may be converted enzymatically into the keto acid by deamination or transamination. The amide does not participate in these reactions but appears to play a rôle in glycolysis (14), ammonia transport, and possibly protein synthesis.

#### SUMMARY

*S-n*-Propyl-, *n*-butyl-, *n*-amyl-, and *n*-hexylhomocysteine and the corresponding sulfoxides and sulfones were prepared and the antimetabolite activity of the latter against glutamic acid was compared with that of the sulfoxides and sulfones derived from methionine and ethionine. The sulfoxide and sulfone derived from ethionine are the weakest inhibitors in both series. With increasing alkyl chain length, growth inhibition tends to become more pronounced both by the sulfoxides and sulfones. These inhibitions, however, are not completely reversed by glutamine. The dissociation constants of the sulfoxides and sulfones with the widest divergence in antimetabolite activity, *i.e.* those derived from methionine and ethionine, were found to be identical.

<sup>1</sup> Recently, we reported bacterial growth inhibition by oxalacetate and bicarbonate which was overcome by traces of glutamine or increased amounts of glutamic acid (11). In those experiments, in accordance with others (8), it was found that at a glutamic acid concentration of 40  $\gamma$  per ml. *Lactobacillus arabinosus* grew as well at an initial pH of 8 (adjusted before autoclaving) as at lower pH ranges. However, there is no growth at all even in 72 hours if the pH is adjusted after autoclaving. The discrepancy is due to the large pH change from pH 8 to 7.1 in the medium during autoclaving. Traces of glutamine ( $1 \times 10^{-5}$  M) or increased glutamate concentration overcome the inhibiting effect of the high pII of the medium. Since we find that the bacteria do not grow at these pH levels in media adjusted after autoclaving, the inhibitory effect of oxalacetate and its decomposition product, bicarbonate, must be ascribed to their alkalizing effect. The inhibition by the high pII is overcome not only by traces of glutamine and by increased glutamate but also by sulfhydryl (cysteine, homocysteine, thioglycolic acid).

$\alpha$ -Aminopimelic acid is ineffective as an antimetabolite.  $\beta$ -Hydroxyglutamic acid is a potent antimetabolite against glutamic acid in *Lactobacillus arabinosus* with an antibacterial index of about 30. This inhibition is reversed by small amounts of glutamine or increased amounts of glutamic acid. This result indicates that  $\beta$ -hydroxyglutamic acid acts like the sulfoxide derived from methionine in blocking the amidation of glutamic acid.

We wish to thank Mrs. E. Wainfan and Mr. H. K. Miller for their able assistance during the course of this work.

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# STUDIES ON CHOLESTEROL ESTERASES

## I. ENZYME SYSTEMS IN RAT TISSUES\*

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(Received for publication, June 24, 1948)

It has long been known that cholesterol esters are formed and broken down in the animal body, but little work has been done on the sites and mechanisms of these transformations. In 1912, Schultz (6) performed the first valid experiments, and showed that cholesterol esters could be hydrolyzed by liver extracts. Repetition of this work by Mueller (4) yielded no evidence of hydrolysis, but seemed to indicate a synthesis of cholesterol esters from free cholesterol on incubation.

Shope (7) in 1928 tested extracts of guinea pig tissues, with use of the natural cholesterol ester in serum as a substrate, and found that all tissues showed some hydrolytic activity, although liver, kidney, and muscle were the most active. His work was criticized by Sperry (8), who had demonstrated a cholesterol-esterifying system in blood serum. In the absence of non-incubated control levels of cholesterol in Shope's work, it was just as plausible to suggest an inhibition of normal serum-esterifying activity by tissue extracts as to postulate a hydrolytic system for cholesterol esters in the extracts. The possibility of the simultaneous existence of both esterifying and hydrolytic systems was not advanced.

Klein, in an accurate study of esterase activity of tissue extracts (2), found that, at pH 5.3, saline extracts of liver would split 70 to 85 per cent of the serum cholesterol esters in 15 hours. Such activity was noted in liver, spleen, kidney, adrenal, and intestinal mucosa. At this pH, the esterifying system of the serum itself is inactive.

Two important papers have appeared pointing to a possible rôle of the intestinal wall. In the first of these, Mueller (5) showed that the lymph of cholesterol-fed dogs contained a constant ratio of free to esterified cholesterol, regardless whether the lipide was fed in the free or esterified form.

\* The material reported here is part of a thesis submitted by Marie L. Nieft in partial fulfillment of the degree of Doctor of Philosophy in Biochemistry in the Graduate School of the University of Southern California.

The authors wish to express their appreciation for the use of the facilities of the Hancock Foundation.

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This was evidence for the existence of both esterifying and hydrolytic enzymes, either in the gastrointestinal secretions or in the intestinal wall. Secondly, Fröhlicher and Süllmann (1) observed a marked absolute increase in the cholesterol esters of canine chyle after feeding cholesterol in triolein, although the relative proportions of free and esterified cholesterol varied widely.

In view of these scattered but significant data, it has seemed valuable to us to attempt a study of the systems responsible for formation and hydrolysis of cholesterol esters in the rat. We have found that there are two opposing systems which are not different aspects of one reversible process. The apparent activity of any given crude preparation represents the balance between two distinct reactions.

### *Methods*

*Tissue Extracts*—Male albino rats from our stock colony were used, and ranged in weight from 250 to 350 gm. Except in the experiments noted, they were kept on the regular stock diet previous to use. The animals were anesthetized with nembutal and the tissues promptly removed and chilled in ice water. The livers were rinsed and the intestines were slit and washed in cold water before being ground with sand in a mortar with an equal weight of 0.9 per cent sodium chloride solution. The crude extract was centrifuged lightly and the supernatant liquid poured off through gauze for immediate assay.

*Assay Techniques*—Assays were carried out in 15 ml. test-tubes containing 0.5 ml. of tissue extract, 0.5 ml. of 0.05 M succinate or phosphate buffer of such a pH that the final value was 6.5, and 1.0 ml. of the substrate. The substrates, cholesterol and cholesterol palmitate, were prepared either in dioxane solution or in colloidal aqueous suspension. These devices were necessary because of their extreme insolubility in water. The presence of 50 per cent dioxane in the mixture during incubation did not seem to inhibit enzyme activity, as judged by comparison with the activity against aqueous colloidal substrates.

The assay tubes were stoppered and placed in a 37° incubator for 8 to 18 hours before extraction and determination of cholesterol. All assays were done in duplicate.

*Preparation of Colloidal Suspensions*—Cholesterol or cholesterol palmitate was dissolved in warm acetone, and this solution was poured dropwise into rapidly boiling water, with constant stirring. The final suspension was allowed to cool before filtering through paper.

*Cholesterol Determination*—The assay tubes, containing 2.0 ml. of solution, were extracted with 10.0 ml. of a 3:2 alcohol-ether mixture, added forcibly from a calibrated syringe. The restoppered tube was allowed to



stand for 10 minutes, centrifuged quickly, and suitable aliquots taken into 15 ml. centrifuge tubes for the determination of free and total cholesterol. The following method is a modification of the original Schoenheimer and Sperry method as proposed by Chaney.<sup>1</sup>

All aliquots were evaporated to dryness at 60° by means of a gentle stream of air, and redissolved in 3 ml. of 1:1 acetone-alcohol mixture. The samples for total cholesterol were hydrolyzed with 2 drops of 30 per cent aqueous KOH at 60° for 30 minutes, then made acid to phenolphthalein with 15 per cent acetic acid. 1 ml. of 0.5 per cent digitonin in 50 per cent alcohol was added to all tubes, and they were then incubated at 35° for 3 hours. The digitonide was well packed by centrifugation, the supernatant carefully decanted, and the precipitate washed with 3 ml. of anhydrous ether. The mixture was again centrifuged and the ether decanted. The precipitates were dried at 60° and dissolved in 0.5 ml. of glacial acetic acid. 3 ml. of chloroform were added, and the stoppered tubes warmed to 35° in a solid aluminum test-tube block equipped with a heater and thermostatic control. Then 1.0 ml. of color reagent was added to each tube. (This color reagent was prepared by adding 1 part of concentrated H<sub>2</sub>SO<sub>4</sub> to 9 parts of acetic anhydride cooled in an ice bath.) After exactly 10 minutes at 35° the tubes were transferred to the ice bath for another 10 minute period. They were then read in the Klett colorimeter against a reagent blank, with a 660 m $\mu$  filter.

We have found that with this technique duplicate determinations of up to 0.6 mg. of cholesterol consistently check within 0.01 mg.

#### RESULTS AND DISCUSSION

*Liver*—Both esterification of free cholesterol and splitting of cholesterol esters have been demonstrated in saline extracts of rat livers. As may be seen by comparison of the data in Tables I and II, which show the activity of the same extracts in both systems, it is impossible to tell beforehand which will be the predominant reaction in any given crude extract. Therefore, since we presumably have present two opposing systems, the data can be interpreted only in a qualitative rather than a strict quantitative sense. Nevertheless, with the figures contained in Tables I and II, several facts can be discovered regarding these systems.

It is immediately apparent from Table I that some component of soya lecithin augments the action of the hydrolytic system. The rôle of the added lecithin cannot be to render the substrate soluble, for both cholesterol and cholesterol esters are completely soluble in the final 50 per cent dioxane mixture at the concentrations used.

<sup>1</sup> Chaney, A. L., unpublished work.

Hydrolytic action can occur in either phosphate or succinate buffers, but the esterification system seems to function better when phosphate ion is added. This is shown by Experiments 408 and 430 of Tables I and II, which are representative of four such experiments. The process of esterification may involve some intermediary phosphate compound. Preliminary tests with the addition of adenosine triphosphate and glucose-1-phosphate to the system have yielded negative results.

Experiment 508 in Table II, typical of four others, shows that in the esterification process tristearin and oleic acid may be used just as easily

TABLE I  
*Hydrolysis of Cholesterol Palmitate by Liver Extracts*

Experiment No.	Buffer	Soya lecithin	Cholesterol values				Freed
			Control		Incubated		
			Total	Free	Total	Free	
			mg.	mg.	mg.	mg.	mg.
408	0.05 M succinate	+	0.43	0	0.43	0.02	(0.02)
	After dialysis, 0.05 M succinate	+	0.60	0	0.60	0.06	0.06
414	0.05 M phosphate	—	0.60	0	0.60	0	0
	0.05 “ succinate	—	0.60	0	0.60	0.02	(0.02)
	0.05 “ phosphate	+	0.60	0	0.60	0.12	0.12
	0.05 “ succinate	+	0.60	0	0.60	0.10	0.10
421	0.05 “ phosphate	—	0.60	0	0.60	0.05	0.05
	0.05 “ “	+	0.60	0	0.60	0.14	0.14

These results are indicative of five experiments. Experiment 421 was included to show the occasional positive results without lecithin in crude preparations. This effect has never been seen in purified preparation. Assay mixture, 0.5 ml. of rat liver extract in saline; 0.5 ml. of buffer, final pH of mixture 6.5; 1.0 ml. of dioxane, containing cholesterol palmitate and 5 mg. of soya lecithin as noted. Incubation, 21 hours, 37°. The values in parentheses indicate results not considered significant.

as palmitic acid. The use of cholesterol alone as substrate gives a small amount of esterification, presumably because of the presence of small amounts of fatty acids or fatty acid donors in the crude preparation. The normal liver lipase is still active and may well account for the fact that tristearin is utilizable as a fatty acid donor.

The work with liver extracts was considerably hampered by the fact that these extracts are capable of destroying cholesterol, that is, rendering it unprecipitable with digitonin, under much the same conditions as those used here. This is apparent in Table III, where we see that the values for total cholesterol after incubation are lower than the controls. This effect is absent after the material has been dialyzed. This aspect of

TABLE II  
*Esterification of Cholesterol by Liver Extracts*

Experiment No.	Fatty acid source	Buffer	Cholesterol values				Esterified
			Control		Incubated		
			Total	Free	Total	Free	
			mg.	mg.	mg.	mg.	mg.
408	Palmitate	0.2 M phosphate	0.62	0.62	0.62	0.34	0.28
	"	0.05 " succinate	0.62	0.62	0.62	0.58	0.04
	After dialysis, palmitate	0.05 " "	0.62	0.62	0.62	0.62	0
	" "	0.05 " phosphate	0.62	0.62	0.62	0.59	0.03
414	Palmitate	0.05 " "	0.60	0.60	0.60	0.24	0.36
421	"	0.05 " "	0.50	0.50	0.48	0.48	0
430	"	0.05 " "	0.67	0.67	0.67	0.60	0.07
	"	0.05 " succinate	0.67	0.67	0.67	0.67	0
508	"	0.05 " phosphate	0.65	0.65	0.65	0.58	0.07
	Tristearin	0.05 " "	0.67	0.67	0.67	0.60	0.07
	Oleate	0.05 " "	0.67	0.67	0.67	0.60	0.07

Assay mixture, 0.5 ml. of rat liver extract in saline; 0.5 ml. of buffer, final pH of mixture 6.5; and 1.0 ml. of dioxane containing cholesterol and fatty acid source in equimolar ratios. Incubation, 21 hours, 37°.

TABLE III  
*Stability of Esterifying System to Dialysis and Lyophilization*

Preparation	Buffer	Control		Incubated		Esterified
		Total	Free	Total	Free	
		mg.	mg.	mg.	mg.	
Crude Extract	0.05 M phosphate	0.41	0.41	0.38	0.31	0.07
Lyophilized	0.05 " "			0.36	0.31	0.05
Dialyzed vs. 0.2 M phosphate	None			0.41	0.36	0.05
Dialyzed vs. 0.9% NaCl	0.2 M phosphate			0.41	0.36	0.05
Dialyzed vs. distilled water	0.2 " "			0.41	0.41	0

This experiment is typical of many. Assay mixture, 0.5 ml. of rat liver extract in saline or buffer; 0.5 ml. of buffer or saline, final pH of mixture 6.5; and 1.0 ml. of dioxane containing 0.41 mg. of cholesterol and an equimolar amount of palmitic acid. Incubation, 21 hours, 37°.

cholesterol metabolism was also treated by Marx and Lipsett (3). However, even in cases in which the cholesterol recovery is not complete after incubation, the fact that some esterification took place is undeniable, al-

though it must be remembered that the figures represent only a minimal value.

In Table III it is noted that the esterifying system of liver is stable both to lyophilization and to dialysis against 0.9 per cent NaCl. Dialysis against distilled water has been found to lead to irreversible inactivation of the system. These findings have been repeatedly checked.

TABLE IV  
*Esterification and Hydrolytic Reactions of Extracts of Rat Small Intestine*

Experiment No.	Preparation	Substrate form	Hydrolysis				Freed	Esterification				Esterified
			Control		Incubated			Control		Incubated		
			Total	Free	Total	Free		Total	Free	Total	Free	
			mg.	mg.	mg.	mg.		mg.	mg.	mg.	mg.	mg.
1204	Crude	Dioxane	0.48	0.15	0.48	0.17	(0.02)	0.20	0.19	0.20	0.14	0.05
		Colloid	0.48	0.19	0.48	0.19	0	1.12	1.12	1.08	0.91	0.19
	Dialyzed vs. water	"	0.56	0.07	0.56	0.12	0.05	1.12	1.03	1.12	1.02	(0.01)
106	Crude	"	0.52	0.19	0.52	0.19	0	1.12	1.12	1.12	1.02	0.10
	Dialyzed vs. water, lyophilized	"	0.40	0.10	0.40	0.14	0.04	1.12	1.12	1.12	1.12	0
930	Crude	Dioxane	0.32	0.02	0.32	0.11	0.09					
	Heated at 100°, 15 min.	"	0.32	0.02	0.32	0.02	0					

These tissue extracts were from animals fed 1 per cent lanolin in the diet for 16 to 21 days before the experiments. Similar results have been obtained in four of five preparations. The remaining one showed no activity after dialysis. Assay procedure, 0.5 ml. of intestinal extract, in saline; 0.5 ml. of buffer, final pH of mixture 6.5, phosphate buffer for esterification and succinate buffer for hydrolysis; and 1.0 ml. of substrate, either as dioxane solution or as aqueous colloidal suspension. For hydrolysis, cholesterol palmitate and 5 mg. of soya lecithin; for esterification, cholesterol and 3 mg. of sodium palmitate. Incubation, 8 hours, 37°. The values in parentheses indicate results not considered significant.

*Intestine*.—Similar types of enzyme activity were obtained with extracts from the small intestine of the rat, as is shown in Table IV. However, it is interesting that esterification has been demonstrated only when the animals had been fed a diet containing 1 per cent lanolin (which contains cholesterol esters) or 1 per cent cholesterol for 2 or 3 weeks previous to sacrifice. Such animals are represented in Experiments 1204 and 106. Here it is seen that the extract initially showed only esterifying activity, since all tests for hydrolytic action against cholesterol esters were negative. These tests were carried out not only against the dioxane solutions, but also against colloidal suspensions of the ester in water.

Dialysis of the crude extract against distilled water for 24 hours evidently inactivates the esterifying system, for after this procedure, esterification cannot be demonstrated even though the phosphate ion is restored. In addition, the hydrolytic system can now be detected in the dialyzed extract. This would seem to indicate that in these crude extracts the hydrolytic system is masked by the synthetic system, and that only after the latter has been removed can a true indication of hydrolytic activity be obtained.

Heating the enzyme extract to 100° before addition of the substrate has always destroyed the hydrolytic activity. Representative figures for one such experiment are seen in Experiment 930, Table IV. Experiment 106 is presented as one example of the repeated observation of the stability of the hydrolytic system of lyophilization.

TABLE V  
*Effect of Substitutions for Soya Lecithin in Hydrolytic System*

Substance added	Cholesterol freed
	mg.
5 mg. lecithin.....	0.08
2 " monoacetin.....	0
4 " diacetin.....	0
2 " inositol.....	0
4 " triacetin.....	0

Assay mixture, 0.25 ml. of water-insoluble enzyme from crude extract of rat intestine, equal to 1 gm. of fresh tissue; 0.25 ml. of water-soluble fraction cofactor from crude extract, equal to 1 gm. of fresh tissue; and 1.0 ml. of cholesterol palmitate colloid, containing 0.9 mg. of cholesterol palmitate. Incubation, 18 hours, 37°.

The rôle which soya lecithin plays in the hydrolytic system is obscure. The main components of this substance have been reported as lecithin, cephalin, oil, phytosterol, inositol, and carbohydrates. Carbohydrate may be ruled out as an active factor, because an alcoholic extract of soya lecithin has the same activity as the crude material.

It is improbable that phytosterols have any vital rôle in the enzyme systems of the animal body, and those sterols present cannot contribute false values for free cholesterol, because non-incubated controls are negative. Inositol and triglycerides cannot be substituted for soya lecithin, as may be seen in Table V. Therefore, it is possible that lecithin or one of the cephalins may be the active component. In an attempt to evaluate the rôle in this system, it was decided to see whether soya lecithin could be replaced by other chemically similar compounds. Assays with glycerol monoacetate and glycerol diacetate instead of soya lecithin proved negative.

It need not be thought that this is some extraneous component which is being added to the hydrolytic system, for it is observed that the crude undialyzed extracts do not always require supplementary lecithin. After the system has been purified by thorough dialysis or salt fractionation, the activation of the hydrolytic system by lecithin is most marked. In these purified preparations, activity has never been observed in the absence of lecithin.

Whether or not the two systems described are identical between tissues remains to be proved. The requirements for esterification and hydrolysis are sufficiently unusual to make it likely that similar systems are involved in both liver and intestine.

#### SUMMARY

1. The existence of an enzyme system which can esterify cholesterol has been verified in rat liver and intestine. This system requires the presence of phosphate ion and a fatty acid source.

2. The splitting of cholesterol esters can be accomplished by extracts of rat liver and intestine. This hydrolytic activity is accentuated by the presence of soya lecithin.

3. These two systems are not aspects of one reversible process.

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## STUDIES ON CHOLESTEROL ESTERASES

### II. CHARACTERIZATION OF THE HYDROLYTIC SYSTEM FROM INTESTINAL MUCOSA\*

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(Received for publication, June 24, 1948)

In a previous report (1), an enzyme system from rat intestine which will split cholesterol esters was described. It is the purpose of this paper to characterize this system and its component parts more fully.

#### EXPERIMENTAL

The assays for hydrolytic activity were carried out as described previously, usually with colloidal cholesterol palmitate as the substrate, and the incubation mixture supplemented with 5 mg. of soya lecithin.

In the course of attempting to purify this hydrolytic system, it became apparent that we were dealing with at least two factors. As may be seen from Table I, exhaustive dialysis against distilled water separated two components, one water-soluble, the other water-insoluble. Assay of the two fractions, separated by centrifuging, showed that either fraction alone was inactive, although together they exhibited the original activity of the preparation. The prolonged dialysis would occasionally lead to inactivation of the preparation, but when electrodialysis was adopted, with the consequently shortened time interval, this difficulty disappeared.

Since the solubility of the one component resembled that of the globulins, a successful attempt was made to separate the two factors by ammonium sulfate fractionation (Table II). The crude extract was diluted with 1 volume of distilled water and cooled to 2° in an ice bath. Then, with constant stirring, the calculated amount of saturated ammonium sulfate solution was added dropwise, the mixture allowed to stand for a few minutes, and the precipitate collected by centrifuging in chilled tubes. The precipitate was dissolved in water and dialyzed until ammonia-free before assay. The volume of the recovered supernatant fluid was measured before the solution was again chilled and brought to the next higher con-

\* The material reported here is part of a thesis submitted by Marie L. Niefert in partial fulfillment of the degree of Doctor of Philosophy in Biochemistry in the Graduate School of the University of Southern California.

The authors wish to express their appreciation for the use of the facilities of the Hancock Foundation.

† Student research fellow of the Life Insurance Medical Research Fund.

centration of ammonium sulfate by the same technique. In this way, successive fractions were separated from the crude tissue extract. The final saturation was carried out with solid ammonium sulfate.

TABLE I  
*Fractionation of Hydrolytic System by Dialysis*

Preparation	Cholesterol freed mg.
Control (non-incubated).....	0
Crude extract.....	0.09
Dialyzed, water-soluble fraction.....	0
“ water-insoluble fraction.....	0
“ both fractions.....	0.10

These results are typical of those in ten similar experiments. The tissue source in these experiments was the small intestine of the rat. Dialysis was carried out against cold distilled water until precipitation of the globulin fraction occurred. Assay mixture, 0.5 ml. of tissue extract or equivalent; 0.5 ml. of 0.05 M succinate buffer, final pH 6.5; and 1.0 ml. of dioxane containing 0.6 mg. of cholesterol palmitate and 5 mg. of soya lecithin. Incubation, 8 hours, 37°.

TABLE II  
*Separation of Components of Cholesterol Esterase by Ammonium Sulfate Fractionation*

Precipitated between 0 and 50 per cent saturation	Precipitated between 50 and 100 per cent saturation	Soluble at 100 per cent saturation	Cholesterol freed mg.
+	+	+	0.04
+	—	—	0
—	—	+	0
+	—	+	0.05
+	+	—	(—0.01)
—	+	+	0
—	+	+	0

The tissue source in this experiment, which is typical of three others, was the small intestine of the rat. Assay mixture, 1.0 ml. of 0.05 M succinate buffer, pH 6.6, containing an amount of lyophilized dialyzed fraction equivalent to 1 ml. of original extract; and 1.0 ml. of dioxane containing 0.6 mg. of cholesterol palmitate and 5 mg. of soya lecithin. Incubation, 8 hours, 37°. The values in parentheses indicate results not considered significant.

The composition of the “cofactor,” the water-soluble component required for this reaction, presents itself as an interesting problem. The fact that it is both non-dialyzable and soluble in saturated ammonium sulfate seems to fix its molecular weight between rather narrow limits. Hence, a study was made of the heat stability of the two components



(Table III). Heating to 100° for 15 minutes before assaying completely inactivated the water-insoluble "enzyme" fraction, but an unheated enzyme preparation exhibited the original activity when combined with heated or unheated cofactor.

The relative concentrations necessary for maximal activity of the two factors were of interest in deciding whether activity of the heat-stable "cofactor" was due to its actual participation in the enzyme system or whether it was serving in some manner as one of the reactants. Accordingly, the activity of a given amount of enzyme prepared by dialysis was assayed against progressive dilutions of one of the purest preparations of the cofactor. The latter was prepared by saturation of the extract with ammonium sulfate, filtration, and dialysis and lyophilization of the super-

TABLE III  
*Heat Stability of Hydrolytic Enzyme and Cofactor Fractions Prepared from Rat Intestine by Dialysis*

Enzyme*	Cofactor*	Cholesterol freed
		mg.
+	+	0.05
+, H	+	(0.01)
+	+, H	0.05
+, H	+, H	0
+	—	0
—	+	(0.01)

Substrate, 1.0 ml. of cholesterol palmitate colloid, containing 0.80 mg. of ester plus 5 mg. of soya lecithin.

\* + indicates the presence of a fraction; H indicates that the fraction was heated to 100° for 15 minutes, then cooled before addition of substrate.

The values in parentheses indicate results not considered significant.

natant. The results are shown in Fig. 1. Here, when the concentration of the cofactor falls below 1 mg. per ml. (1 mg. corresponding to the amount from 138 mg. of tissue), the activity of the mixture falls off. Some activity is noted until the concentration reaches 0.025 mg. per ml. Below this level the results are no longer significant, although they indicate a still further decrease in activity. The amount of enzyme used in this series corresponded to that derived from 400 mg. of wet tissue and weighed approximately 3.5 mg. Part of this weight is due to sodium chloride, since the preparation was lyophilized from saline solution. In view of the extremely small amounts of cofactor needed, as well as the fact that the optimal ratio between enzyme and cofactor is approximately that found in the tissue extract, it appears that the cofactor is a part of the enzyme system rather than one of the reactants.

The determination of the optimal pH for the hydrolytic reaction was also of interest. This procedure was impossible with crude preparations, because it was not feasible to buffer the mixture strongly enough to prevent a marked drifting toward the acid side as the incubation proceeded. When purer preparations were available, however, the experiment was tried, and the results are shown in Fig. 2. With 0.05 M phosphate buffers, there was still some drift toward the acid side during the 18 hour incubation period, but it was very much less marked. The pH of the final mixture was

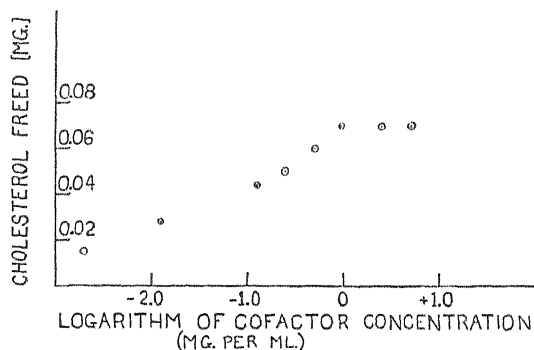


FIG. 1. Limiting cofactor concentration for activity

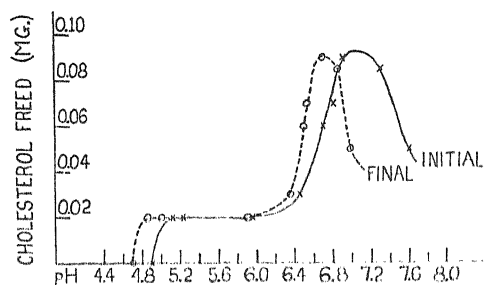


FIG. 2. Optimal pH for hydrolytic activity. Substrate, colloidal cholesterol palmitate; incubation, 18 hours at 37°.

taken before incubation, and duplicate tubes were incubated. At the end of that time, the pH was again recorded and the contents of the tube saved for analysis of free and total cholesterol. The activity curves when plotted against the pH were seen to be very similar whether the initial or final pH was used. Therefore the optimal pH for the hydrolysis of cholesterol palmitate under these conditions is seen to lie between 6.7 and 7.1. Incubation of the substrate alone through the experimental pH range led to no splitting of the ester.

The stability of the cofactor toward acid and alkali was studied on a

lyophilized preparation made from rat tissue by ammonium sulfate saturation of the aqueous tissue extract. Treatment with 3 per cent aqueous KOH at 100° for 15 minutes destroyed the activity of the cofactor. Similar treatment for 30 minutes with 6 N HCl left the activity of the preparation unchanged. It was found, however, that after acid hydrolysis the active component became dialyzable through cellophane.

Chemical data on an impure compound mean little, but it is worthy of note that, on the ammonium sulfate preparation described above, a maximal content of 1 per cent carbohydrate (orcinol method) and of 2.1 per cent tyrosine (Folin-Ciocalteu) were found. Work is proceeding toward identification of this cofactor.

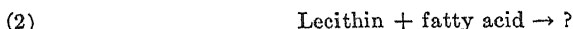
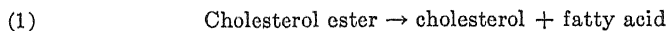
An attempt was made to study the reaction in other species. An aqueous tissue extract from rabbit intestine exhibited hydrolytic activity toward cholesterol palmitate, but on salt fractionation, the portions soluble and insoluble in 50 per cent saturated ammonium sulfate both retained activity. It was not possible to separate two mutually dependent components, as in the rat. However, heat inactivation of the supernatant liquid from tissue extract half saturated with ammonium sulfate, followed by assay of this material against a cofactorless rat enzyme preparation, showed that the rabbit extract contained a substance which could substitute for rat cofactor. There was undoubtedly another esterase in the crude rabbit extract which obscured the measurement of the system in which we were interested.

A similar experiment with cat intestine showed that the precipitate from half saturation with ammonium sulfate exhibited no activity unless the fraction soluble in saturated ammonium sulfate was present. The latter was inactive by itself. Cross reactions between rat and cat preparations showed that the two systems are mutually interactive, although their absolute identity is not proved.

#### DISCUSSION

While most of the lipases and esterases thus far studied exhibit some degree of activity toward the higher molecular weight alcohols and acids, none has ever been shown to require a cofactor for such activity. In addition, most of these hydrolytic reactions can be shown to reach definite equilibrium points, approachable from either direction (2), which is not the case with this system. It is evident that we have here a more complex reaction.

The rôle of lecithin was discussed in the previous report (1). It may be that we are actually dealing with two reactions,



in which the first is driven to the right by a constant removal of the liberated fatty acids. If this is valid, however, reaction (1) should proceed to some extent even without the help of lecithin. This has not been observed, although the possibility exists that it could occur to the extent of about 4 per cent, which represents the lower limit of sensitivity of the assay used (equivalent to 0.02 mg. of cholesterol freed from 0.50 mg. present as the ester).

For the sake of simplicity in discussing the experimental work, the assumption has been made that the cofactor is needed in reaction (1) above. However, evidence is not yet sufficient to decide whether the cofactor is necessary as an adjunct to the esterase performing the actual hydrolysis or whether it takes part in some later reaction, as, for example, reaction (2). Nevertheless, it is evident that the hydrolysis of cholesterol esters cannot be detected in the absence of this cofactor, although the limits of experimental error already discussed do not rule out the possibility.

#### SUMMARY

1. The hydrolytic system for cholesterol esters in the intestinal tissue of the rat has been studied, and found to consist of at least two factors.

2. One component is a globulin type protein, heat-labile, and precipitated by 50 per cent saturation with ammonium sulfate.

3. The other component is a heat-stable, non-dialyzable substance soluble in saturated ammonium sulfate. The minimal concentration of this substance necessary for activity and some of its chemical characteristics have been investigated.

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## EXCRETION OF CREATININE AND CREATINE BY BEEF STEERS\*

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(Received for publication, July 31, 1948)

In the classical theory of protein metabolism proposed by Folin (1) two types of protein metabolism are recognized, one a constant or endogenous metabolism, and the other a variable or exogenous metabolism. According to this theory the endogenous metabolism is represented by the excretion of creatinine at a relatively constant rate which is independent of protein intake. More recently, as pointed out by Friedemann and associates (2), there has been increased emphasis on the variation rather than on the uniformity of creatinine excretion, and the independence of creatinine excretion of protein intake has been questioned. Most of the research relating to this problem has been conducted with human subjects and laboratory animals.

The creatinine and creatine excretion of dairy cattle on standard dairy rations was studied by Ashworth and Brody (3); however, no data were presented to show the effect of protein intake on the excretion of these two urinary constituents. Carpenter (4) determined the creatinine and creatine excretion of beef steers on pasture and on maintenance rations. The protein intake of the animals was not calculated. Although similar values for creatinine excretion were obtained in these two investigations, slightly higher creatine values were reported for the dairy cows than for the steers. In a comparison of the results, Ashworth and Brody (3) suggest that creatine excretion in cattle is influenced by the level of protein intake.

The present study was undertaken to determine the creatinine and creatine excretion of beef steers on rations containing different amounts of protein and different amounts of dietary urea added as a protein substitute (5).

### EXPERIMENTAL

Hereford steers, 2 years old, in metabolism stalls were fed rations of constant composition during 20 day periods. During the last 10 days of each period urine was quantitatively collected by means of rubber funnels

\* This study was supported in part by a research grant from E. I. du Pont de Nemours and Company, Inc.

TABLE I  
Daily Values for Total N, Creatinine N, and Creatine N in Urine of Beef Steers Receiving Supplements of Cottonseed Meal and Urea-Containing Pellets

Steer No.	1			2			3			4			5			6		
	Urea-containing pellets†	(a)	(b)	Cottonseed meal	(a)	(b)	Urea-containing pellets†	(a)	(b)	Cottonseed meal	(a)	(b)	Urea-containing pellets†	(a)	(b)	Urea-containing pellets†	(a)	(b)
Supplement fed*																		
Average body weight, kg.	345.8			371.5			332.9			353.6			307.5			290.8		295.9
N intake, gm.	124.0			117.5			124.2			117.8			124.0			124.2		117.8
Day of determination	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)
1	53.9	2.62	1.26	51.3	3.62	0.00	57.5	2.29	0.47	45.8	3.58	0.84	58.5	3.54	1.01	66.0	1.60	1.23
2	60.0	3.39	1.11	57.7	3.62	1.71	57.0	2.15	0.94	54.7	3.29	1.26	68.6	4.02	0.85	43.7	1.65	1.90
3	58.1	3.28	0.73	51.9	4.09	0.41	62.4	2.51	1.93	53.4	3.36	1.31	66.6	3.92	0.87	45.4	2.39	0.69
4	67.8	5.53	1.30	57.7	4.86	0.43	82.4	3.85	1.37	56.2	4.50	0.45	69.5	5.12	0.44	62.2	2.37	0.65
5	62.0	3.72	1.00	57.9	3.83	0.48	63.2	3.23	0.60	50.8	3.51	1.32	68.6	3.85	0.72	48.1	2.29	0.26
6	54.8	4.74	0.93	57.3	5.47	0.18	76.2	3.82	1.06	56.0	3.65	0.85	64.8	3.99	1.62	46.9	1.08	0.46
7	56.3	4.96	1.40	54.0	4.99	0.75	68.9	3.44	0.77	46.5	3.52	1.67	50.2	2.27	0.82	44.5	2.12	1.05
8	60.9	3.64	0.79	51.3	3.66	1.36	66.5	2.79	1.29	47.1	2.96	1.45	76.4	3.71	1.19	61.3	2.00	0.84
9	62.4	3.69	2.00	53.5	4.64	0.66	37.5	1.95	0.12	49.0	3.77	0.39	73.8	4.02	0.49	37.7	1.82	0.48
10	63.6	3.63	0.95	60.0	4.48	0.10	90.8	1.78	0.38	59.7	4.02	1.43	76.1	3.92	1.79	88.6	5.39	0.20
Average	60.0	3.92	1.15	55.3	4.33	0.61	66.2	2.78	0.89	51.9	3.62	1.07	67.3	3.84	0.98	54.4	2.27	0.77
Creatinine coefficient	11.24			11.63			8.36			10.25			12.48			7.80		9.88

(a), (b), and (c) represent total urinary N, creatinine N, and creatine N, respectively, in gm.

\* All steers received 10 pounds of prairie hay and 3 pounds of supplement daily.

† The urea pellets contained 75 per cent cottonseed meal, 4 per cent urea, 10 per cent molasses, and 11 per cent hominy feed. Urea supplied 18.8 per cent (23.3 gm.) of the total nitrogen intake of Steers 1, 3, 5, and 6.

which led to glass collection jars beneath the floor of the stalls. Toluene was used as a preservative and sufficient sulfuric acid was added to the collection jars to maintain the urine below pH 6. Preliminary work showed that at values below pH 6 both creatinine and creatine are stable. Urine was collected and measured daily. Creatinine and creatine were determined by Folin's method as outlined by Hawk *et al.* (6).

In the first series of experiments the nitrogen intake of all of the steers was approximately the same, the rations being composed of prairie hay supplemented with either cottonseed meal or a pelleted feed containing 75 per cent cottonseed meal, 4 per cent urea, 10 per cent molasses, and 11 per cent hominy feed. Creatinine and creatine were determined on daily samples of urine. The results are presented in Table I.

TABLE II

*Average Creatinine and Creatine Nitrogen Excretion by Beef Steers on Rations Containing Different Amounts of Protein and Urea Nitrogen*

Daily N intake*		No. of animals	Average body weight	Daily creatinine N excreted	Daily creatine N excreted	Average creatinine coefficient	Average creatine coefficient
Total	As urea						
gm.	gm.		kg.	gm.	gm.		
53.8	0.0	4	332.8	3.39	0.32	10.19	0.96
61.6	7.8	4	326.0	3.16	1.11	9.69	3.40
95.7	0.0	4	336.2	3.68	0.54	10.95	1.61
96.0	24.0	24	318.5	3.72	0.97	11.68	3.05
100.0	0.0	8	317.5	3.67	0.89	11.56	2.80
117.7	0.0	3	340.7	3.63	0.84	10.65	2.47
119.2	23.5	4	336.3	3.94	0.45	11.72	1.34
124.0	23.3	4	320.0	3.20	0.94	10.00	2.97

\* All steers received 10 pounds of prairie hay supplemented with varying amounts of cottonseed meal, urea, molasses, and hominy feed daily.

In the second series of experiments, the nitrogen intake of the steers was progressively increased from about 54 gm. daily to 124 gm. daily. The rations were composed of prairie hay supplemented with different amounts of the pelleted feed constituents used in the first experiments. Creatinine and creatine were determined on 10 day pooled samples of urine which had been preserved as described above and stored at 0° during the collection period. The combined results representing data secured from 55 animals are presented in Table II.

#### RESULTS AND DISCUSSION

The results in Table I show that the excretion of total nitrogen and creatinine nitrogen of individual steers on a uniform nitrogen intake is

relatively constant from day to day as compared to the differences between individuals. There are, however, some apparent exceptions. The greatest variations were observed with Steers 3 and 6 on the 9th and 10th days of collection. Such variations as were observed in the daily creatinine excretion of individual animals were for the most part unrelated to the daily total nitrogen excretion. Creatine excretion varied markedly from day to day.

Differences between animals in excretion of total nitrogen over the 10 day periods were related to small differences in the amount, and particularly in the form, of nitrogen ingested. As might be expected total nitrogen excretion of steers that received urea was generally high. There were also marked differences between animals in creatinine excretion. The differences were unrelated to the amount and form of nitrogen ingested. Although creatinine excretion is generally related to body weight, in these experiments the steers with low creatinine excretion values had correspondingly low creatinine coefficients.

Average creatinine and creatine coefficients<sup>1</sup> for steers ingesting from 53.8 to 124.0 gm. of nitrogen daily are given in Table II. The nitrogen balance status of these animals varied from nitrogen equilibrium to a positive nitrogen balance of 22.3 gm. daily. It is obvious from the results that changes in nitrogen intake over the range studied and the addition of urea to the rations were without effect on the creatinine coefficients. The weighted mean creatinine coefficient was 11.18. This value is higher than the value of 9.5 reported by Ashworth and Brody (3) for dairy cows. Differences in breed, age, and sex of the animals might contribute to this difference. Carpenter (4) reported creatinine coefficients between 8 and 9 for steers; however, destruction of creatinine during storage and shipping of the urines as described in his paper might easily account for these lower values.

The creatine coefficients shown in Table II varied from 0.96 to 3.40 and could not be correlated with total nitrogen intake. The weighted mean creatine coefficient was 2.62, a value similar to that reported by Carpenter (4) but considerably lower than the value of 7.6 reported by Ashworth and Brody (3) for dairy cows.

#### SUMMARY

The daily excretion of creatinine nitrogen by beef steers is relatively constant and is unaffected by changes in the protein content of the ration or by the addition of urea to the ration as a protein substitute.

<sup>1</sup> In Tables I and II and in the text, creatinine and creatine coefficients refer to the mg. of creatinine N and creatine N, respectively, excreted per kilo of body weight per day.



There appears to be an individual variation in the amount of creatinine excreted by steers of similar breed, age, and body weight.

The average creatinine coefficient of 2 year-old Hereford steers ingesting from 54 to 124 gm. of nitrogen daily is 11.18.

Creatine excretion by steers is variable.

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# THE EFFECT OF GELATIN ON THE TRANSFORMATION OF TRYPTOPHAN TO NIACIN IN RATS ON LOW CASEIN DIETS\*

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(Received for publication, August 19, 1948)

It was demonstrated by Elvehjem and collaborators (1-4) that, if to a niacin-free diet containing 9 per cent casein and permitting moderate growth there was added an excess of tryptophan-deficient proteins (corn, gelatin, acid hydrolysates of proteins) or certain amino acids (glycine, threonine, phenylalanine), growth of young rats was impaired or stopped. Furthermore, this effect could be abolished equally well by the addition to the diet of approximately 1.5 mg. per cent of niacin or of 50 mg. per cent of tryptophan. This observation appears to imply that the superimposition of certain amino acids upon a diet containing barely adequate amounts of complete protein resulted in a niacin deficiency.

The question which still remained to be solved was whether the added amino acids raised the quantitative requirements for niacin, or, since tryptophan in the casein is the only known source of niacin in this diet, whether the transformation of tryptophan to niacin is impaired under these conditions. This question is partly answered by the data of Elvehjem *et al.* (4) who showed that the growth of rats receiving the basal 9 per cent casein diet amounted to 12 gm. per week, while the gain in rats receiving the same diet plus 1.5 mg. per cent of niacin was 16 gm. per week. Other data in the published studies of the Wisconsin group and our own unpublished data support the view that niacin added to a low protein diet enhances growth to a small extent, probably sparing a part of the food tryptophan as well as supplying the vitamin. This is further borne out by the growth data on the rats receiving 9 per cent casein plus added amino acids in which the addition of 1.5 mg. per cent of niacin restored, or even improved, the rate of growth, as compared with animals receiving the basal diet and the added niacin only ((4) Table I).

\* Grants in aid of this investigation from the United States Public Health Service, the Nutrition Foundation, Inc., the John and Mary R. Markle Foundation, and the Duke University Research Council are gratefully acknowledged. The DL-tryptophan used in these studies was furnished by the Sterling-Winthrop Research Institute, through the kindness of Dr. M. L. Tainter, Director.

† Nutrition Foundation, Inc., Fellow.

The experiments described below furnish further evidence that the capacity of the organism to form niacin from tryptophan is impaired when a low protein (10 per cent casein-sucrose) diet is supplemented with a large amount of a tryptophan-free protein (gelatin).

#### EXPERIMENTAL

Weanling male albino rats of the Vanderbilt strain were kept singly in metal metabolism cages with tray bottoms. Fresh food and water were supplied daily and the rats were weighed at semiweekly intervals. Each rat was offered an adequate amount of food daily, and on the following day the residue was weighed and discarded. The basal diet used in these studies contained casein 10, sucrose 83, salts 4 (5), cottonseed oil 3, choline 0.5, L-cystine 0.4 gm., supplemented with 1 mg. each of thiamine, riboflavin, and pyridoxine, 6 mg. of Ca pantothenate, 15 mg. of inositol, 0.02 mg. of biotin, 0.02 mg. of folic acid, and 0.10 mg. of vitamin K per 100 gm. 2 drops of cod liver oil were given each week. The gelatin added to the diet was at the expense of the sucrose.

Urine was collected in 72 hour periods under 1 ml. of toluene and 0.5 ml. of glacial acetic acid. The urine and washings were made to a total volume of 50 ml., and after filtering, a suitable aliquot was taken from each of the six urines in the group, and the pooled sample stored in the refrigerator. The urinary excretion of N<sup>1</sup>-methylnicotinamide was determined by the fluorometric acetone condensation method (6).

While the animals ingesting the basal diet (10 per cent casein), Group 1, and the casein + gelatin diet supplemented by niacin or tryptophan, in Groups 5 and 6, developed no outward symptoms of deficiency, the rats in Groups 2, 3, and 4 developed in the course of the first 3 weeks of the unsupplemented casein + gelatin diet rough coats, severe diarrhea, and incrustations of brownish red pigment about the mouth and paws. The addition of 5 mg. per cent of niacin to the diet produced remission of all of these symptoms; the addition of 50 mg. per cent of DL-tryptophan produced a temporary remission of the diarrhea only, while the other symptoms persisted and cleared gradually after the large 25 mg. dose of L-tryptophan.

The data in Table I and Fig. 1 show the growth-depressing effect of 6 per cent gelatin added to the 10 per cent casein diet. They also demonstrate that the addition of either 5 mg. per cent niacin or 50 mg. per cent of tryptophan at the beginning of the experiment is equally effective in abolishing this inhibitory action of gelatin. It is worth noting that the addition of the niacin or of the tryptophan to the gelatin-containing diet (Groups 5 and 6) results in better growth than on the basal diet alone without gelatin (Group I). Thus, the growth-depressing effect of gelatin appears to be due almost entirely to a lack of niacin, and not to any other-

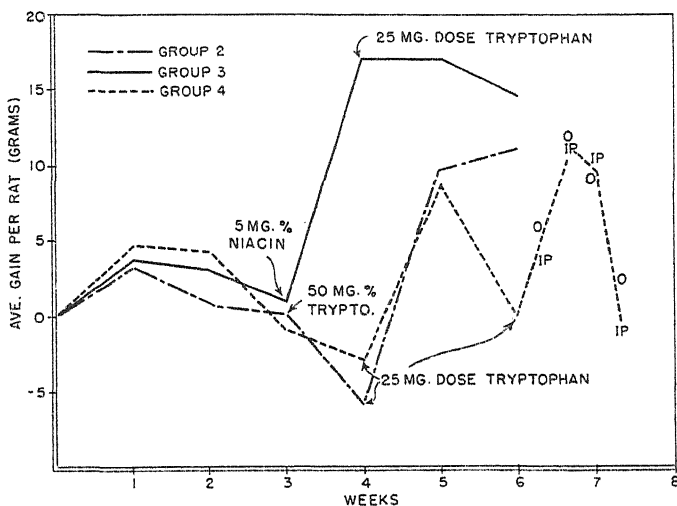


FIG. 1. Growth responses of rats in Groups 2, 3, and 4 maintained for the first 3 weeks on a 10 per cent casein + 6 per cent gelatin diet. Beginning in the 4th week, to the diet of Group 2 were added 50 mg. per cent of DL-tryptophan and to the diet of Group 3 were added 5 mg. per cent of niacin; the diet of Group 4 remained unchanged. At the beginning of the 5th week all animals received a single 25 mg. dose of L-tryptophan intraperitoneally. At the beginning of the 7th week, three animals in Group 4 were given another 25 mg. dose of L-tryptophan orally (O), and three animals received the same amount intraperitoneally (IP).

TABLE I  
*Modifying Effects of Niacin and Tryptophan upon Growth Inhibition  
Produced by Gelatin in Rats*

The average values for six rats in each group, the gain being measured in gm. per rat per week.

Average gain	Group 1. Basal 10 per cent casein	Group 2. Basal + 6 per cent gelatin	Group 3. Basal + 6 per cent gelatin	Group 4. Basal + 6 per cent gelatin	Group 5. Basal + 6 per cent gelatin, 5 mg. per cent niacin	Group 6. Basal + 6 per cent gelatin, 50 mg. per cent DL-tryptophan
<i>per wk.</i>						
1-3rd	8	1	2	3	16	18
4th	9	-6*	17†	-3	16	15
5th-6th	13‡	11‡	16‡	4‡	12‡	11‡

\* 50 mg. per cent of DL-tryptophan added to diet.

† 5 mg. per cent of niacin added to diet.

‡ On the first day of the 5th week all the animals received a single dose of 25 mg. of L-tryptophan intraperitoneally.

wise toxic effect of the large amount of gelatin in the diet. Table I further shows the striking differences in response to supplements of niacin and

tryptophan added to the casein-gelatin diet *in the beginning of the experiment* (Groups 5 and 6), as compared with the results obtained when these supplements were added *after 3 weeks of progressing growth depression* produced by the gelatin (Groups 2 and 3). Whereas the animals in Groups 5 and 6 continued to grow well (15 to 18 gm. per week), the rats in Group 2, when given a supplement of 50 mg. per cent of tryptophan in the diet, actually lost weight to about the same extent as the control Group 4, which received no supplement to the gelatin diet. On the other hand, the animals in Group 3, when supplemented with 5 mg. per cent of niacin, showed an immediate and large gain in weight (Fig. 1).

At the beginning of the 5th week all animals received intraperitoneally a single dose of 25 mg. of L-tryptophan. The rats in Groups 2 and 4 showed an immediate positive gain in weight. The animals in Group 2 which were receiving 50 mg. per cent of tryptophan showed a larger gain and maintained it during the next 2 weeks (Fig. 1), whereas the control animals in Group 4 (casein-gelatin diet alone) responded with an increase in weight during the first 6 days after the injection and then declined again. As shown in Fig. 1, the animals in Group 4 were given at the beginning of the 7th week another single dose of 25 mg. of L-tryptophan, three rats receiving the dose orally and three intraperitoneally. All animals showed about equally rapid growth responses during the first 5 days, but lost weight just as rapidly in the next 4 days. These results confirmed those observed previously in the same animals in the 5th week, and also demonstrated that the effect of tryptophan is the same, regardless of the mode of administration.

The excretion of N<sup>1</sup>-methylnicotinamide was measured in all groups for the 3 day period following the single intraperitoneal dose of 25 mg. of L-tryptophan at the beginning of the 5th week. The control urines were collected during the preceding 3 day period. The results summarized in Table II indicate that in Group 4 the effect of the gelatin in the diet is to depress the excretion of extra N<sup>1</sup>-methylnicotinamide after the test dose of tryptophan. The addition of niacin to the diet of the animals in Group 3 restores the excretion of extra N<sup>1</sup>-methylnicotinamide to the level found in Group 1. However, the animals in Group 4 supplemented with tryptophan did not respond to the test dose. In Groups 5 and 6, where supplements of niacin and tryptophan, respectively, were given from the beginning of the experiment, significantly large increases in the excretion of N<sup>1</sup>-methylnicotinamide were observed. The reason for the different responses in Groups 5 and 6 is not apparent.

These data indicate clearly that the presence of an excess of gelatin in the 10 per cent casein diet results in an impairment of the metabolic pathway from tryptophan to niacin. This defect, if permitted to develop for several

weeks, cannot be remedied by the addition of a small amount of tryptophan (50 mg. per cent) but can be abolished either by adding a small amount of niacin or by relatively very large amounts of tryptophan (25 to 100 mg. daily).

TABLE II  
*Excretion of N<sup>1</sup>-Methylnicotinamide in Rats Fed Low Protein Diet containing 6 Per Cent Gelatin and Supplements of Niacin and Tryptophan*

Group No. (6 rats each)	Diet	Average excretion of N <sup>1</sup> -methylnicotinamide per 100 gm. rat	
		Control	Increase over control value after 25 mg. L-tryptophan intraperitoneally
		$\gamma$ per 72 hrs.	$\gamma$ per 72 hrs.
1	Basal (10% casein)	41	465
2	" + 6% gelatin ( + 50 mg.% DL-tryptophan, beginning 4th wk.)	80	75
3	Basal + 6% gelatin ( + 5 mg.% nicotinic acid, beginning 4th wk.)	344	456
4	Basal + 6% gelatin	60	72
5	" + 6% gelatin + 5 mg.% nicotinic acid	316	764
6	" + 6% gelatin + 50 mg.% DL-tryptophan	52	252

#### DISCUSSION

Presumably the growth-depressing effect of whole corn, corn proteins, acid-hydrolyzed proteins, gelatin, or amino acids such as glycine, DL-threonine, or DL-phenylalanine (4), when added to a low casein diet, is primarily due to the ensuing biochemical defect in the metabolic relationship of tryptophan to niacin in rats. In all cases described in the literature and in our observation, this defect is restored by small amounts of niacin alone. While added tryptophan may produce somewhat enhanced growth as compared with niacin, it is apparent that the major effect of tryptophan is due to its yield of niacin, and the further increment in growth may be due to the availability of extra tryptophan for tissue synthesis in animals kept on a tryptophan-poor diet. From this viewpoint, the pellagragenic effect of an excess of corn in the diet is explicable on two accounts: the deficiency of tryptophan as a source of niacin, and the imbalance of amino acids leading to an impairment in the transformation, tryptophan  $\rightarrow$  niacin, in a diet which is already poor in niacin.

Schweigert and Pearson (7) recently reported that the addition of 12 per cent gelatin to a 12 per cent casein-sucrose diet depressed the growth of

rats and that the addition of 1 mg. per cent of niacin or the feeding of 100 mg. doses of DL-tryptophan daily restored the growth. It is to be noted that this restoration was obtained with relatively huge doses of tryptophan. In the present study, while this effect of large doses of free tryptophan is confirmed, the inadequacy of smaller amounts of tryptophan equivalent to concentrations to be found in diets containing 15 to 20 per cent casein in overcoming the syndrome in niacin-depleted rats is clearly demonstrated.

#### SUMMARY

The inhibition of growth and of the transformation of tryptophan to niacin, as determined by the urinary excretion of N<sup>1</sup>-methylnicotinamide after a test dose of tryptophan, in young rats on a diet containing 10 per cent casein and 6 per cent gelatin can be adequately prevented by the initial inclusion of either 5 mg. per cent of niacin or 50 mg. per cent of tryptophan. If the same amount of tryptophan is added after the animals have been on the above diet for 3 weeks and have developed deficiency symptoms, the growth and the excretion of N<sup>1</sup>-methylnicotinamide remain depressed. The addition of 5 mg. per cent of niacin to the diet after 3 weeks does restore both growth and the excretion of N<sup>1</sup>-methylnicotinamide to normal levels. A single large parenteral or oral dose (25 mg.) of tryptophan restores for a few days only the normal growth curve of the animals. These observations in conjunction with the data of other investigators are interpreted to demonstrate that the major deleterious effect of gelatin, of other tryptophan-deficient proteins, and of amino acids when added to a low casein diet is due to the impairment of the metabolic process involved in the tryptophan-niacin interrelationship.

We wish to thank Miss Betty Jean Peck for technical assistance.

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# IDENTIFICATION OF THE ANTIBIOTIC SUBSTANCE FROM CASSIA RETICULATA AS 4,5-DIHYDROXYANTHRA- QUINONE-2-CARBOXYLIC ACID\*

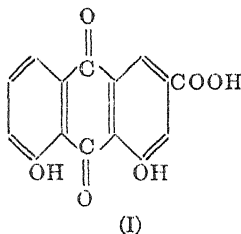
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(Received for publication, June 9, 1948)

Robbins, Kavanagh, and Thayer (1) recently reported the isolation from the leaves of *Cassia reticulata* Willdenow of a substance possessing antibiotic activity *in vitro* against *Bacillus mycoides*, *Bacillus subtilis*, *Staphylococcus aureus*, *Mycobacterium smegmatis*, and *Neisseria gonorrhoeae*. The compound was obtained in crystalline form by careful acidification of its dilute alkaline solution, and was called "cassic acid."

This substance has now been identified as rhein, a compound first isolated from Chinese rhubarb (2) and later from a species of cassia (*Cassia acutifolia*) (3). The structure of rhein has been established as 4,5-dihydroxyanthraquinone-2-carboxylic acid (I) (4-7).



For purposes of comparison, rhein was prepared both by isolation from rhubarb and by synthesis. Identity of the isolated and synthetic samples with the antibiotic substance and with each other was established by mixed melting points of the diacetates of the compounds, as well as by identity of the absorption spectra of the compounds and of their diacetates (Figs. 1 and 2). Rhein from rhubarb and synthetic rhein have the same antibiotic activity<sup>1</sup> as rhein from cassia.

\* This investigation was supported in part by a grant from the Albert H. and Jessie D. Wiggin Foundation.

<sup>1</sup> Antibiotic activity refers to activity against *Staphylococcus aureus*, tested in nutrient broth near pH 7. For details of the method, see Kavanagh (8). The number of dilution units in a sample is given by the total volume (in ml.) to which it can be diluted with nutrient broth and still bring about complete inhibition of the growth of the test organism. The assays were carried out by a geometric serial dilution (2, 4, 8, etc.) and the values therefore lie between those given and the next higher power of 2.

Antibiotic activity of quinones has been reported frequently (9), but relatively few studies have been made in this connection with anthraquinone derivatives. Anthraquinone itself and chrysophanic acid have been found inactive (10), while it is interesting to note that anthraquinone and certain

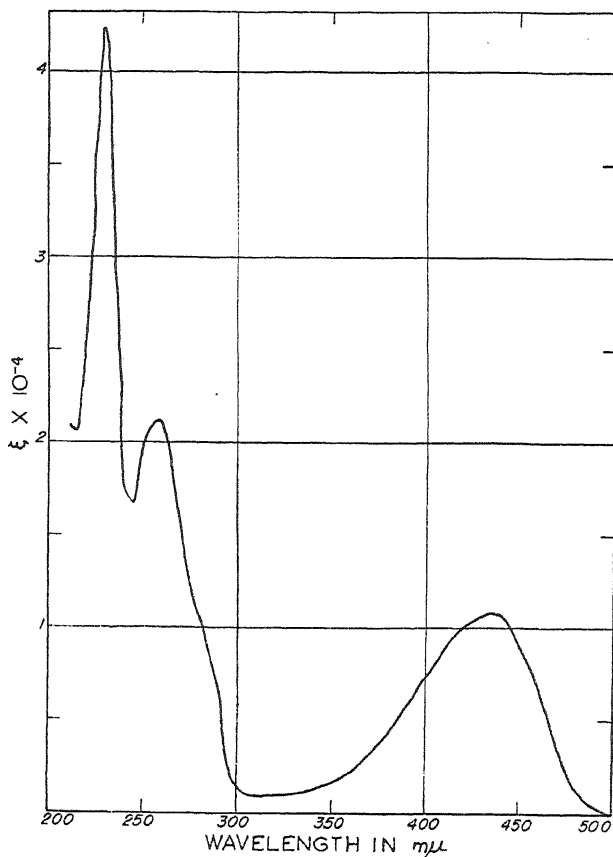


FIG. 1. Rhein in 95 per cent ethanol

of its derivatives, injected intraperitoneally, inhibit the growth of Twort carcinoma in mice (11). Emodin, which is present in rather large amounts in rhubarb, was found to be bacteriostatic under the conditions of the test employed.<sup>1</sup> The antibiotic activity of rhubarb extracts, therefore, is not due entirely to the presence of rhein.

Other anthraquinone derivatives are being prepared for further study.

Compounds of the type of the acetate which are gradually hydrolyzed under physiological conditions (Fig. 3)<sup>2</sup> may also prove of interest.

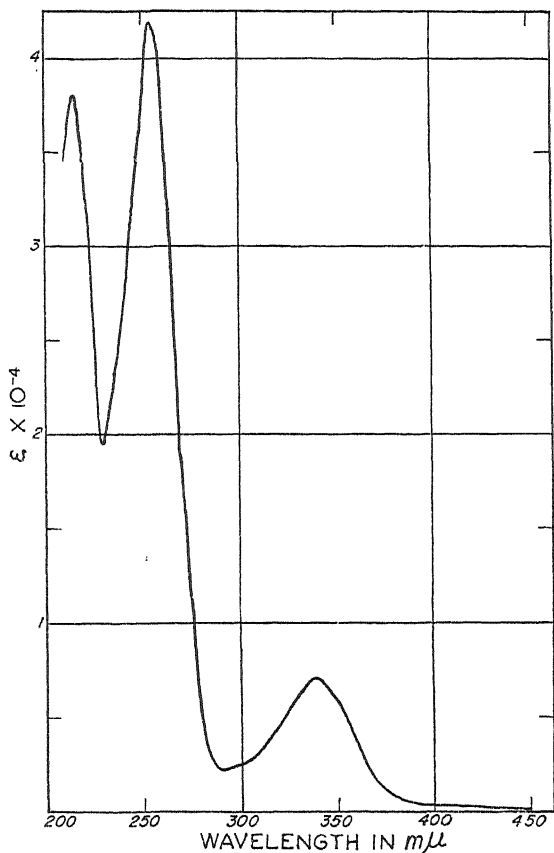


FIG. 2. Rhein diacetate (or its methyl ester) in 95 per cent ethanol

#### EXPERIMENTAL<sup>3</sup>

*Isolation Procedure*—A batch of 100 gm. of coarsely ground leaves of *Cassia reticulata* Willd. was placed loosely in a fluted filter paper over a

<sup>2</sup> Rhein diacetate shows about half the antibiotic activity of rhein itself. However, it is difficult to determine whether this activity is actually due to the diacetate or whether it results from the slow splitting of the diacetate to free rhein which occurs under the conditions of the test. It is apparent from the curves in Fig. 3 that, after 20 hours of incubation, the acetate has been in large part hydrolyzed.

<sup>3</sup> All melting points are corrected.

wad of glass wool in an extractor of the type described by Clarke and Kirner (12), and the active material was extracted for three periods of 1, 1.5, and 1.5 hours, with 750 ml. of water<sup>4</sup> for each extraction. (Extraction by suspension of the leaves in boiling water was unsatisfactory, since a muddy solution resulted with which it was very difficult to work.) The

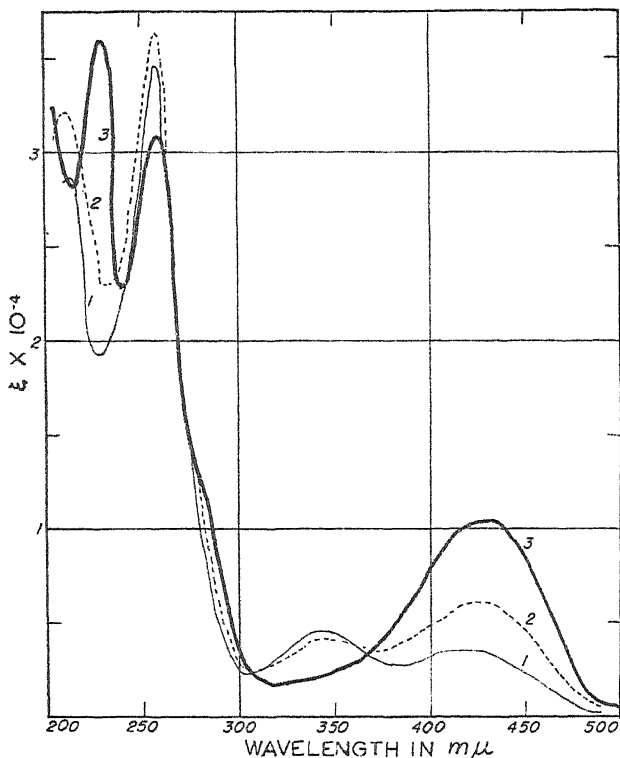


FIG. 3. Rhein diacetate in aqueous solution at a pH near 7. (The substance was dissolved by neutralization with NaOH.) Curve 1, within 1 hour of solution; Curve 2, after 2 hours incubation at 37°; Curve 3, after 20 hours incubation at 37°.

combined solution usually contained about 23,000 dilution units<sup>1</sup> for *Staphylococcus aureus*, of which about two-thirds were obtained in the

<sup>4</sup>Methods involving carbonate or bicarbonate extraction of the leaves yielded mixtures from which the active substance could not satisfactorily be isolated. In one of the earlier large batches (2 kilos) of leaves extracted with carbonate and subjected to a complicated fractionation, a biologically inactive, pale yellow crystalline material was obtained, in amounts roughly corresponding to the expected amount of rhein, while none of the latter could be obtained. The structure of this substance is being investigated.

first extract. The solution was concentrated under reduced pressure to about 100 ml., and the syrupy concentrate was extracted with methyl isobutyl ketone in a continuous extractor until the extract was practically colorless. The organic solution was then shaken in a separatory funnel with small portions (10 to 25 ml.) of 5 per cent sodium bicarbonate solution, as long as the typical reddish color appeared in the extracts. Usually a total of about 50 to 100 ml. was used. The iced bicarbonate extract was acidified to about pH 2 with cold, dilute hydrochloric acid, and the tan amorphous precipitate was centrifuged, washed with water, and dried *in vacuo*. The yield varied from 215 to 348 mg., with an average, in twenty-three runs, of 310 mg., having a potency of 64 dilution units<sup>1</sup> per mg. This amounts to about 20,000 dilution units, or 87 per cent of the 23,000 in the original aqueous extracts.

On recrystallization from acetic acid, the yields varied widely, depending on the length of time the material was in contact with the hot solvent. Previous removal of dark pigment with cold acetic acid, or with acetone followed by cold acetic acid, was necessary for subsequent successful crystallization. The active substance crystallized in pale yellow hair-like needles, mostly in rosettes or sheaves. Under certain conditions, usually too concentrated a solution or too sudden crystallization from the hot solution, an orange powder precipitated, which consisted of microscopic diamond-shaped spicules. This had the same antibiotic activity as the yellow needles. The melting point was not characteristically different. Exact conditions for production of each form were not determined, but seeding the solution with the yellow needle form usually resulted in crystallization of this type. Likewise, local cooling usually started crystallization in the yellow form, which then continued. The recovery of activity varied from roughly 100 per cent to as low as 6 per cent, and the potency of the once crystallized material from 128 to 256 dilution units per mg. The substance is difficult to obtain pure. Samples, recrystallized several times and dried *in vacuo* at 100°, melted at 326–329° with decomposition, and gave the following analyses:

$C_{15}H_{25}O_6$ .	Calculated.	C 63.37, H 2.81
284	Found.	" 62.78, 63.73; H 3.22, 3.28

The absorption spectra in 95 per cent ethanol and in 0.1 N sodium hydroxide are shown in Figs. 1 and 4 respectively. The ethanol solution shows maxima at 230, 260, and 430  $m\mu$ , and minima at 215, 245, and 307  $m\mu$ . In alkali the spectrum changes gradually with time, as shown in Fig. 5. The measurements for the sodium hydroxide curve in Fig. 4 were completed within half an hour after the sample was dissolved, and show maxima at 240 and 500  $m\mu$ , and a minimum at 370  $m\mu$ . Between 265 and 280  $m\mu$ , there is a distinct shoulder.

The crystalline material gives the tests described as characteristic for rhein (13).

*Diacetate of Rhein Isolated from Cassia (4,5-Diacetoxyanthraquinone-2-carboxylic Acid)*—A solution of 142 mg. (0.5 mm) of crystalline rhein and 200 mg. of dry sodium acetate, in 25 to 50 ml. of acetic anhydride, was

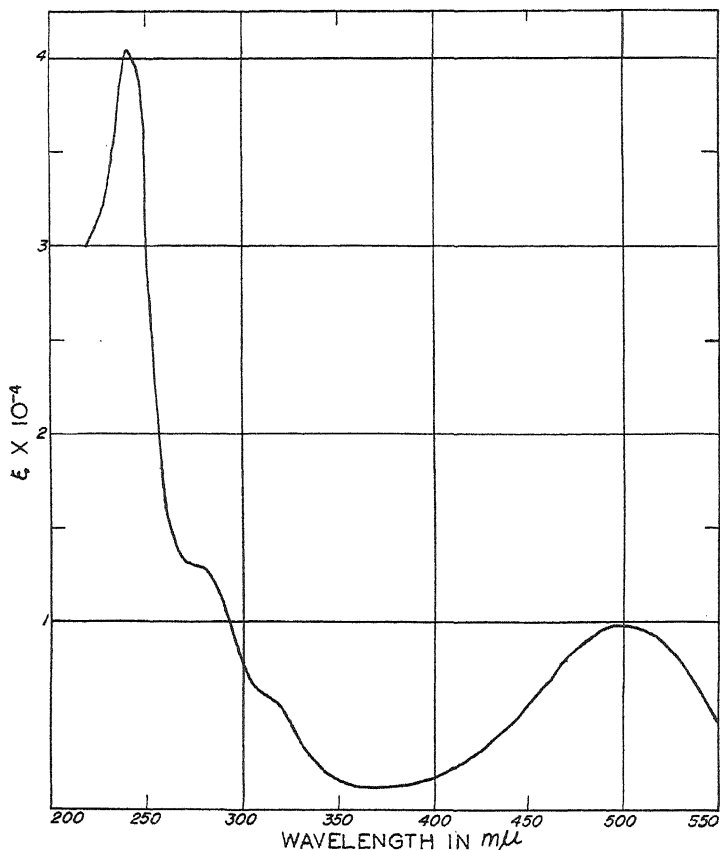


FIG. 4. Rhein in 0.1 N NaOH at room temperature. (The "blank" cell of the Beckman spectrophotometer contained 0.1 N NaOH.)

boiled under a reflux for 15 minutes, centrifuged clear of sediment, and poured into 250 ml. of ice water. The precipitate, a pale yellow powder, melted unsharply around 236°. After one recrystallization from acetic acid, 110 mg. (60 per cent of the theoretical) were obtained, melting at 247–252°. After several recrystallizations, the material melted at 250–

251°, and gave no depression with the synthetic product melting at 250–253°.

The absorption spectrum in 95 per cent ethanol is shown in Fig. 2. Maxima are present at 215, 255, and 340  $m\mu$ , and minima at 230 and 295  $m\mu$ . The curve for the synthetic diacetate is superimposable.

The solution for the curves shown in Fig. 3 was prepared by neutralization of the diacetate with sodium hydroxide. While the diacetate is much

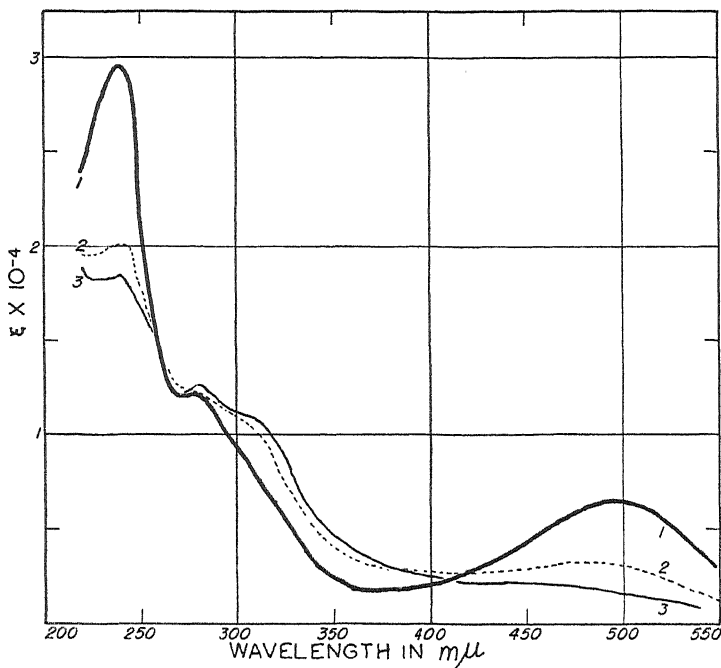


FIG. 5. The effect of alkali on rhin. (The conditions are the same as those for Fig. 4.) Curve 1, after 4 hours; Curve 2, after 48 hours; Curve 3, after 4 days.

more readily crystallized than rhin itself, a good analysis could not be obtained. A summary of the difficulties described by previous authors is given by Tutin and Clewer (3), who believed that they had the pure compound, but remarked on its peculiar behavior; *i.e.*, it dissolved completely in hot xylene and reprecipitated immediately. In a later paper (14), Tutin attributes this behavior to the presence of acetic anhydride of crystallization.

The results of analyses of samples prepared from isolated rhin are presented in Table I. The analytical figures suggest retention of the solvent.

*Methyl Ester of Diacetate of Isolated Rhein (4,5-Diacetoxyanthraquinone-2-carboxylic Acid, Methyl Ester)*—A suspension of 55.2 mg. (0.15 mm) of the diacetate in dry ether was treated with an ethereal diazomethane solution until no further reaction occurred. The ether was removed, and the residue was taken up in 10 ml. of 95 per cent ethanol and a small amount of solid removed by centrifugation. When the alcoholic solution was concentrated to about 3 ml. and allowed to cool, the product crystallized in beautiful clusters of pale yellow needles. Yield, 39 mg. (68 per cent of the theoretical), melting around 184°. After several recrystallizations from 95 per cent ethanol, the product melted at 194–195°. It was dried *in*

TABLE I  
*Analyses of Diacetate of Samples of Rhein Isolated from Cassia*

Sample solvent	Melting point °C.	Dried at	C	H	COCH <sub>3</sub>
			per cent	per cent	per cent
Calculated, C <sub>19</sub> H <sub>12</sub> O <sub>8</sub>	368		61.93	3.26	23.36
Acetic acid	250–251	78°, 1 hr., <i>in vacuo</i>	61.10	3.81	25.35
95% ethanol	252–254	Room temperature, 2 wks. <i>in vacuo</i>	60.85	3.28	21.82

*vacuo* at 100° for 1 hour, for analysis. The absorption spectrum in 95 per cent ethanol is the same as that of the diacetate, shown in Fig. 2.

C <sub>20</sub> H <sub>14</sub> O <sub>8</sub> .	Calculated.	C 62.82, H 3.69, OCH <sub>3</sub> 8.10
382	Found.	" 62.61, " 3.64, " 8.35

*Synthesis of Rhein*—Synthetic rhein was obtained from chrysophanic acid synthesized according to the method of Eder and Widmer (7). The chrysophanic acid was converted to rhein through the acetate, by chromic acid oxidation, as described by Fischer, Falco, and Gross (4). The synthetic rhein melted at 325–330°. Its diacetate melted at 250–253°, and, when mixed with the acetate of the isolated product melting at 250–251°, it gave no melting point depression. Absorption spectra of the rhein samples and of their diacetates were identical (Figs. 1 and 2). Both the synthetic and isolated rhein samples showed antibiotic activity of 256 dilution units per mg.

*Isolation of Rhein from Rhubarb Root*—A 50 gm. batch of dry, coarsely ground root of *Rheum officinale* (rhubarb root, U. S. P., Chinese, Penick) was extracted as described for cassia leaves for four 1.5 hour periods, with one 1 liter and three 750 ml. portions of water. The combined extracts contained 46,400 dilution units. The solution was concentrated under reduced pressure to a volume of 400 ml., made 0.25 N with respect to hydro-



chloric acid, boiled under a reflux for 1 hour, and extracted with methyl isobutyl ketone in a continuous extractor for about 12 hours. The organic layer was shaken with 5 per cent sodium bicarbonate solution. On acidification, 204 mg. of crude rhein were obtained, with a potency of 64 dilution units per mg. After purification, the potency was 256 dilution units per mg. The acetate of this material melted at 249–251°, and gave no melting point depression with the acetate of rhein isolated from cassia leaves. The absorption spectra of the two compounds were identical with those of the isolated material and its diacetate, shown in Figs. 1 and 2 respectively.

#### SUMMARY

Simple methods for the isolation of the antibiotic principle from the leaves of *Cassia reticulata* Willd., and from rhubarb root, are described. The antibiotic substance, previously named "cassia acid," is identified as 4,5-dihydroxyanthraquinone-2-carboxylic acid (rhein). A new derivative of rhein, the methyl ester of the diacetate, is described, and absorption spectra are presented for this compound as well as for rhein and its diacetate.

The author wishes to express deep appreciation to Dr. William J. Robbins for his kind encouragement, and also to thank Mrs. Gloria Levenstein for technical assistance, Mrs. Catherine Amos and Miss Corrine Massoth for performing the microbioassays, Mr. William Saschek (College of Physicians and Surgeons, Columbia University, New York) and Mr. Joseph Alicino (The Squibb Institute for Medical Research, New Brunswick, New Jersey) for carrying out the microanalyses, and Dr. Julian Wolff for preparing the figures presented in this paper.

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# A NEW COLORIMETRIC METHOD FOR THE DETERMINATION OF SERUM PROTEIN

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(Received for publication, July 15, 1948)

The method for the determination of serum proteins presented in this communication has certain advantages over other methods now in use. It is simple, accurate, and determines both total protein and albumin. It employs a readily available inorganic standard and may be applied to grossly lipemic as well as normal sera.

## Reagents—

*5 per cent trisodium phosphate solution.* Dissolve 50 gm. of analytical grade trisodium phosphate,  $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ , in 900 ml. of water and make up to 1 liter.

*23 per cent sodium sulfate.* Dissolve 230 gm. of analytical grade anhydrous sodium sulfate,  $\text{Na}_2\text{SO}_4$ , in 900 ml. of water and make up to 1 liter. Store at  $37^\circ$ .

*Cupric phosphate.*  $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ , analytical grade powder.

*0.5 per cent diethyl dithiocarbamate.* Dissolve 0.5 gm. of sodium diethyl dithiocarbamate (Eastman Kodak Company) in 100 ml. of water. Store in the refrigerator.

*Stock copper standard.* Dissolve 0.3026 gm. of analytical grade copper sulfate,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , in 900 ml. of water and make up to 1 liter.

*Dilute standard.* Dilute 10 ml. of stock to 100 ml. Each 1 ml. contains 7.7  $\gamma$  of Cu, and for calibration purposes is equivalent to 1 gm. of protein per 100 ml. of serum.

## Procedure

Into each of three conical 15 ml. centrifuge tubes marked total, albumin, and blank, place 8.0 ml. of 5 per cent trisodium phosphate.

To a fourth tube, called the fractionation tube, add 9.5 ml. of 23 per cent sodium sulfate. At the same time add 2.0 ml. of 23 per cent sodium sulfate to the blank.

To the fractionation tube add 0.5 ml. of serum. Mix by inversion and transfer 2.0 ml. of the suspension to the tube marked total. Add 3.0 ml. of ether to the remaining 8 ml. of protein suspension. Stopper with a rubber stopper, shake vigorously, and centrifuge.

Slant the tube, insert a pipette along the lower wall under the globulin cake, remove 2.0 ml. of the lower fluid layer, and transfer to the albumin tube. The fractionation tube is then discarded.

To each of the remaining tubes add approximately 200 mg. of powdered cupric phosphate. Stopper with a rubber stopper and allow to stand for 90 minutes with occasional shaking.

Centrifuge and transfer 0.5 ml. of the supernatant from each tube to a corresponding colorimeter tube containing 20 ml. of water. Add 2 ml. of 0.5 per cent diethyl dithiocarbamate, mix, and read at 440  $m\mu$  (Evelyn colorimeter).

### *Calculations*

The total protein and albumin values may be read directly from a calibration curve or calculated mathematically from a calibration constant ( $R$ ).

*Preparation of Calibration Curve*—Place 0, 2, 4, 6, and 8 ml. of dilute copper standard in a series of colorimeter tubes. Make each up to 20 ml. with water. Add 0.1 ml. of serum to 8 ml. of 5 per cent trisodium phosphate and 2 ml. of 23 per cent sodium sulfate and transfer 0.5 ml. to each tube. Add 2 ml. of diethyl dithiocarbamate, mix, and read at 440  $m\mu$ . Plot a curve with protein concentrations at 0, 2, 4, 6, and 8 gm. per 100 ml. against the logarithm of the colorimeter readings.

*Determination of Calibration Constant ( $R$ )*—Substitute the colorimeter readings ( $G$ ) and the protein concentrations ( $P$ ) in the equation,  $R = P/(2 - \log G)$ , and calculate the various values of  $R$ . The average  $R$  is then substituted in the equation  $P = LR$ , where  $L = (2 - \log G)$ , and this equation is used to calculate the unknown protein.

### EXPERIMENTAL

The method is based on the ability of protein in trisodium phosphate solution to form a soluble copper complex from slightly soluble cupric phosphate. At equilibrium the amount of copper in solution is proportional to the concentration of protein.

*Determination of Copper*—Sodium diethyl dithiocarbamate proved to be the most satisfactory reagent for the determination of copper (1). Turbidity was encountered in pure copper solutions at certain concentration (2), but blood serum is an ideal dispersing agent, a mere trace being sufficient to render an otherwise turbid copper diethyl dithiocarbamate solution crystal-clear.

The spectral transmission curve of copper diethyl dithiocarbamate as determined on a Cenco spectrophotometer showed an absorption peak at 440  $m\mu$ . With an Evelyn colorimeter equipped with a No. 440 filter, a straight line graph was obtained when the logarithm of the per cent transmittance was plotted against varying concentrations of copper. The color is stable for as long as 24 hours and the intensity is not affected by varying the temperature from 15–45°.

*Calculation of Protein from Copper Concentration*—From the Lambert-Beer law may be obtained the equation,  $C = KL$ , where  $C$  is the copper concentration in micrograms and  $L$  is the optical density ( $2 - \log G$ ). The value of  $K$  was calculated as 10.2 for the colorimeter employed.

Since the amount of serum in the final tube is 0.005 ml., the amount of copper which will be brought into solution by 1 gm. of protein is expressed by the equation,  $E = 20 C/P$ , where  $E$  is the copper equivalent in mg. per gm. of protein and  $P$  is the serum protein concentration in per cent.

From  $E = 20 C/P$  is obtained  $E = 204 L/P$  and  $P = 204 L/E$ . The value of  $E$  is constant, as will be shown, so that  $204/E = R$  and  $P = LR$ .

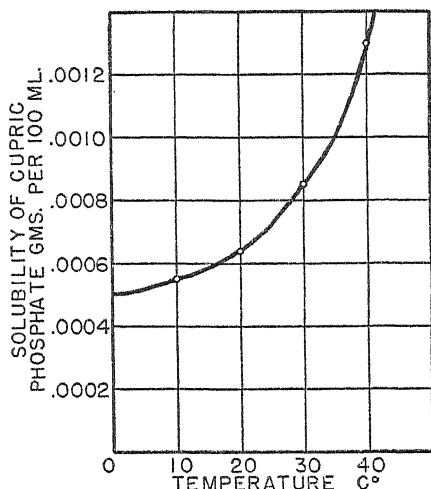


FIG. 1. Solubility of cupric phosphate in 4.0 per cent trisodium phosphate containing 4.6 per cent sodium sulfate.

*Solubility of Cupric Phosphate in Trisodium Phosphate Solution*—Tubes containing 8 ml. of 5 per cent trisodium phosphate and 2 ml. of 23 per cent sodium sulfate were brought to the temperatures indicated in Fig. 1. Approximately 200 mg. of cupric phosphate were then added to each and the tubes allowed to stand with occasional shaking for 90 minutes. After centrifuging, 2.0 ml. of supernatant were removed from each tube and placed in a colorimeter tube containing 18 ml. of water, 0.5 ml. of a 1:100 solution of serum, and 2 ml. of 5 per cent diethyl dithiocarbamate. The results are given in Fig. 1, which shows the solubility of cupric phosphate in trisodium phosphate-sodium sulfate solution at various temperatures. The amount of cupric phosphate which goes into solution is of sufficient magnitude to necessitate the use of a blank.

*Effect of Temperature and Concentration of Protein on Rate of Biuret For-*

mation—Samples of pooled sera on which the protein had been determined by micro-Kjeldahl analysis were subjected to the procedure outlined, but the time and temperature for biuret formation were varied. The results are given in Table I. The time required for the reaction to reach equilib-

TABLE I  
*Effect of Temperature and Protein Concentration on Rate of Biuret Formation*

Protein, Kjeldahl, $P_1$	Temperature	Time	Optical density, $L = (2 - \log G)$	Protein, experimental, $P_2 = 13.2L$	Copper equivalent, $E = \frac{204L}{P_1}$
<i>gm. per cent</i>	$^{\circ}\text{C.}$	<i>min.</i>		<i>gm. per cent</i>	<i>mg. per gm.</i>
1.26	10	5	0.0862	1.14	140
		15	0.0915	1.21	148
		30	0.0942	1.24	153
		45	0.0969	1.28	157
		60	0.0928	1.23	150
		90	0.0996	1.31	161
		120	0.0969	1.28	157
	40	5	0.0862	1.14	140
		15	0.0835	1.10	135
		30	0.0915	1.21	148
		45	0.0969	1.28	157
		60	0.0996	1.31	161
		90	0.0969	1.28	157
		120	0.0955	1.26	155
7.35	10	5	0.4560	6.05	128
		15	0.4690	6.18	131
		30	0.4820	6.36	134
		45	0.5160	6.85	143
		60	0.5380	7.10	151
		90	0.5690	7.50	158
		120	0.5610	7.41	156
	40	5	0.4560	6.05	128
		15	0.4750	6.26	132
		30	0.4950	6.54	137
		45	0.5090	6.70	141
		60	0.5300	7.00	147
		90	0.5610	7.41	156
		120	0.5690	7.50	158

rium is primarily dependent upon the concentration of protein. Temperature, as would be expected, increases the speed of the reaction to some extent, especially with higher concentrations of protein. At 90 minutes a point is reached where the copper equivalent is constant under the conditions studied.

*Proportionality of Copper to Protein*—The protein content of a sample

of pooled sera was determined by micro-Kjeldahl analysis and concentrations of from 1 to 7 per cent were prepared by dilution. These dilute samples were then subjected to the procedure described and the amount of copper combining with the protein calculated. The results are shown in Fig. 2.

*Determination of Copper Equivalent*—The copper equivalent has been defined as the amount of copper in mg. which will combine with 1 gm. of protein under the conditions of the test. This figure may be calculated from the optical density of the final tube in the procedure outlined, if the protein concentration of the serum is determined by another method. Ta-

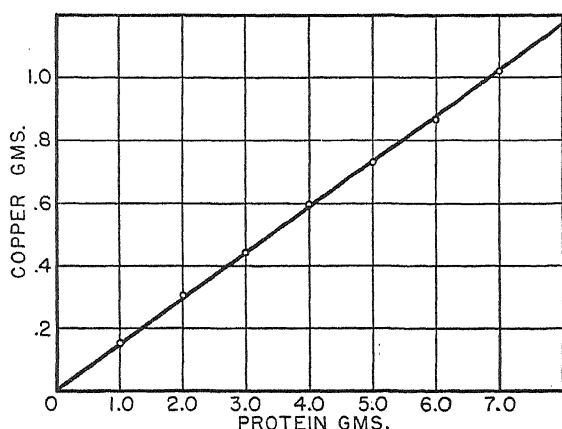


FIG. 2. Relation between concentration of protein and concentration of soluble copper at equilibrium.

ble II gives the copper equivalents as calculated from 50 determinations. The protein was determined by a micro-Kjeldahl procedure utilizing digestion with sulfuric acid and metallic selenium, a modified Parnas-Wagner steam distillation (3), and an acid-alkali titration. Serum fractionation was carried out by the method of Kingsley (4), whose technique is also used for the experimental method. The average value for the copper equivalent ( $E$ ) for both total protein and albumin, as calculated from these figures, is 154.

*Agreement with Kjeldahl Method*—Table II also gives a comparison of the micro-Kjeldahl with the experimental method. The protein concentrations have been calculated by using previously given values for  $K$  and  $E$ , so that  $R = 13.2$ .

*Precision*—In order to determine the precision of the method, five determinations were made on each of seven samples of sera upon which the

TABLE II  
*Copper Equivalents and Agreement with Kjeldahl Method*

Total protein					Albumin				
Optical density, $L = (2 - \log G)$	Copper equivalent, $E = 204 L/P_1$	Protein, Kjeldahl, $P_1$	Protein experimental, $P_2 = 13.2L$	Difference, $P_1 - P_2$	Optical density, $L = (2 - \log G)$	Copper equivalent, $E = 204 L/P_1$	Protein, Kjeldahl, $P_1$	Protein, experimental, $P_2 = 13.2L$	Difference, $P_1 - P_2$
	mg. per gm.	gm. per cent	gm. per cent	gm. per cent		mg. per gm.	gm. per cent	gm. per cent	gm. per cent
0.3100	150	4.20	4.10	+0.10	0.1612	155	2.12	2.14	-0.02
0.4200	140	6.13	5.55	+0.58	0.2840	136	4.26	3.76	+0.50
0.4690	147	6.48	6.20	+0.28	0.3100	143	4.41	4.10	+0.31
0.4820	149	6.60	6.36	+0.24	0.3470	150	4.75	4.60	+0.15
0.4690	154	6.20	6.20	0.00	0.3370	152	4.61	4.45	+0.06
0.2840	156	3.71	3.75*	-0.04	0.1308	157	1.70	1.72	-0.02
0.3570	164	4.42	4.71	-0.29	0.1939	153	2.58	2.56	+0.02
0.2596	176	3.00	3.42*	-0.42	0.1308	179	1.47	1.72	-0.25
0.5530	150	7.51	7.30	+0.21	0.3980	153	5.30	5.26	+0.04
0.5530	154	7.35	7.30	+0.05	0.3370	145	4.75	4.45	+0.30
0.2676	157	3.46	3.52*	-0.06	0.0555	147	0.77	0.73	+0.04
0.3670	154	4.85	4.85	0.00	0.1805	157	2.34	2.38	-0.04
0.4690	136	7.02	6.18	+0.84	0.3370	174	3.94	4.45	-0.49
0.4820	134	7.31	6.36	+0.95	0.2218	151	3.00	2.92	+0.08
0.3670	153	4.90	4.85	+0.05	0.1487	138	2.21	1.96	+0.25
0.4200	165	5.20	5.55*	-0.35	0.2147	155	2.84	2.84	0.00
0.3770	154	4.97	4.97	0.00	0.1739	150	2.36	2.30	+0.06
0.3350	161	4.23	4.40	-0.17	0.1177	158	1.52	1.55	-0.03
0.2924	161	3.70	3.88*	-0.18	0.1487	170	1.78	1.95	-0.17
0.3280	150	4.45	4.34	+0.11	0.1805	166	2.22	2.38	-0.16
0.3980	158	5.14	5.26	-0.12	0.1939	139	2.84	2.56	+0.28
0.4500	162	5.66	5.95	-0.29	0.3080	163	3.85	4.06	-0.21
0.3850	152	5.16	5.10*	+0.06	0.2460	159	3.16	3.24	-0.08
0.3640	159	4.66	4.80	-0.14	0.1939	156	2.54	2.56	-0.02
0.4030	158	5.20	5.32	-0.12	0.1939	158	2.51	2.56	-0.04
Average...	154.2			$\pm 0.226$		154.2			$\pm 0.145$

\* Grossly lipemic sera.

TABLE III  
*Precision of Method and Agreement with Kjeldahl Method As Applied to Clear and Lipemic Sera*

Condition of serum	Fraction determined	Protein, Kjeldahl	Protein, colorimetric method (5 determinations)		
			Minimum	Maximum	Average
Lipemic	Total	3.46	3.42	3.63	3.52
"	"	4.76	4.67	4.94	4.79
"	"	5.86	5.82	6.01	5.87
Clear	"	6.48	6.20	6.51	6.32
"	"	7.50	7.30	7.76	7.48
Lipemic	Albumin	1.76	1.86	2.02	1.98
Clear	"	3.10	2.82	3.02	2.88



protein had previously been determined by the Kjeldahl procedure. The results are given in Table III.

*Effect of Lipemia*—Table III also shows that gross lipemia has little, if any, effect upon the results obtained by this method. In Table II lipemic sera (indicated by the asterisk), as well as clear sera, were used to establish the copper equivalent.

*Specificity*—Obviously the method is not specific for proteins. Any substance having the biuret linkage will give the reaction as well as a number of compounds containing hydroxyl groups and forming soluble copper complexes. Among these are tartrates, citrates, various polyhydroxyl alcohols, and carbohydrates.

From a practical standpoint, however, providing that serum is used so as to rule out the possible effect of an anticoagulant, none of the other substances which might interfere are present in sufficient concentration to affect the reaction.

#### DISCUSSION

By determining the amount of copper held in solution as biuret instead of biuret itself, a sufficient increase in sensitivity is obtained so that any turbidity produced by lipemia is diluted out. By basing the determination on the amount of copper present instead of on the amount of protein, the method may be standardized against copper instead of protein.

#### SUMMARY

A new method for the determination of serum total protein and albumin is presented.

The method is simple and accurate. It is based on an inorganic standard, and may be applied to lipemic as well as normal sera.

The results compare favorably with those obtained by use of the micro-Kjeldahl procedure.

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# ALLOXAN AND DIALURIC ACID: THEIR STABILITIES AND ULTRAVIOLET ABSORPTION SPECTRA

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(Received for publication, July 19, 1948)

It was the purpose of this investigation to determine the ultraviolet absorption spectra of alloxan and dialuric acid. Previous investigators (1-3) reported that these substances had absorption spectra without definite maxima. However, since preliminary studies indicated that maxima were present under certain conditions, it was thought that a more careful investigation should be made.

Although Dixon and Zervas (4) reviewed the literature and concluded that alloxan was stable at pH 7.0, others (5-8) found alloxan to be unstable in neutral and alkaline solutions. Since alloxan decomposed as a first order reaction (9), its absorption spectrum was measured by a determination of the decomposition rate and extrapolation to zero time.

Dialuric acid was reported to be unstable in acid as well as in alkaline solutions, because of its ease of oxidation by dissolved air (1, 7, 8). Since dialuric acid was prepared by the reduction of alloxan with cysteine (6), oxidation of dialuric acid by air was prevented by the addition of cysteine, and the ultraviolet absorption spectrum thus determined. Inasmuch as the ultraviolet absorption of cysteine was low (9), this did not interfere in the measurements.

## EXPERIMENTAL

All measurements were made on a Beckman photoelectric quartz spectrophotometer, with 1 cm. quartz cells.

*Decomposition of Alloxan*—Alloxan (Eastman Kodak) was dissolved in dilute hydrochloric acid solution (1 drop of concentrated hydrochloric acid to 100 cc. of distilled water) in concentrations of 0.05, 0.005, and 0.0005 M. Phosphate buffer (0.17 M) was made up so that on dilution 1:1 with alloxan it would give the desired pH as checked with a glass electrode. Acetate buffer was used at pH 4.0 and 5.0. A mixture of alloxan (concentration depended on wave-length under consideration) and buffer in equal

amounts was then made and the extinction measured at a predetermined wave-length against a blank consisting of a mixture of buffer and water in equal amounts. Readings were taken every half minute after mixing.

The observed extinctions fell rapidly with time. A plot of the logarithm of the observed extinction against time gave a straight line. The slope of this plot was greater at 270  $m\mu$  than at other wave-lengths. Since the total extinction is equal to that of alloxan plus that of the decomposition products of alloxan, the greater slope at 270  $m\mu$  indicated that the contribution of the decomposition products to the total extinction was less at this wave-length than at others. The contribution of the decomposition

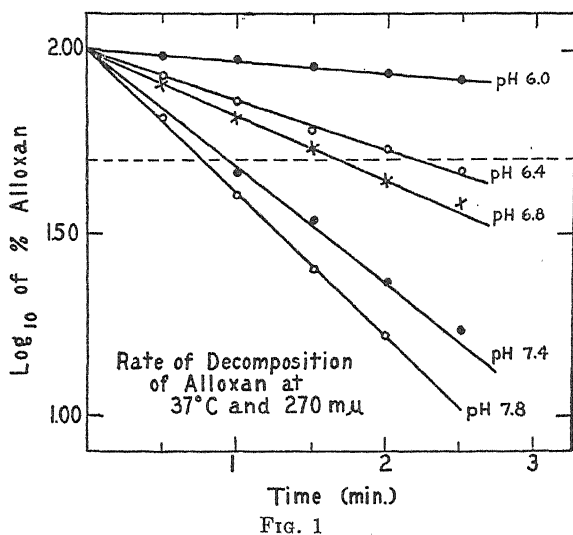


FIG. 1

products to the total extinction should be accounted for in a calculation of the amount of alloxan present at any time. However, when simultaneous equations were set up in the usual manner (10), it was found that the contribution of the decomposition products was small ( $\epsilon = 20$  at 270  $m\mu$ ) and could be neglected during the first 2 or 3 minutes. The rate of decomposition was determined at 37° for several pH values and the results are indicated in Fig. 1. The dotted transverse line indicates the point of 50 per cent decomposition. At pH 7.4 the half life of alloxan was 0.9 minute. At room temperature (about 25°) the rate at decomposition was slower. The half life at pH 7.4 was 2.2 minutes. In 0.5 *N* sodium hydroxide solution the rate was so rapid that there was no change in extinction after the 1st minute.

*Absorption Spectrum of Alloxan*—Since the decomposition of alloxan was

a first order reaction (log of alloxan *versus* time gave a straight line), it was possible to calculate the extinction at zero time. The extinction values for alloxan at different wave-lengths were obtained by extrapolation of the data obtained in the decomposition of alloxan. The results are tabulated in Table I and shown in Fig. 2.

*Absorption Spectra of Dialuric Acid*—Dialuric acid was prepared by the reduction of alloxan with stannous chloride (11). M.p., 224°; N, 19.24 per cent (determined); N, 19.4 per cent (calculated).

TABLE I  
*Absorption Spectra Data on Alloxan*

Wave-length  <i>mμ</i>	Molecular extinction								pK
	10 per cent H <sub>2</sub> SO <sub>4</sub>	pH 4.0	pH 5.0	pH 6.0	pH 6.4	pH 6.8	pH 7.4	pH 7.8	
220					5900	5200	6400	6300	
230				2900	2800	3000	4000	3600	
235	1400	1200	1400	1600	2100	2400	3600	3500	
240	660	650	710	940	1500	2500	3800	3800	6.8
245	330	360	420	770	1500	2500	4800	4800	6.8
250	160	190	230	590	1200	2200	4100	4000	6.8
255	94	120	160	480	1000	1800	3400	3400	6.8
260	92	90	120	348	800	1300	2600	2600	6.8
265	94								
270	90	74	82	150	250	450	980	980	7.0
280	56	44	46	75	98	150	280	270	6.9
290	17	12	14	32	40	58	120	130	6.8
300	2.4		4	15	18	28	52	60	
310						16	22	26	
340							9.8	9.6	

When dialuric acid was placed in buffer at pH 7.4, the extinction at 270 *mμ* fell rapidly as a result of oxidation by dissolved air. Since the specific extinction of dialuric acid was high, extreme dilutions were necessary for measurement in the spectrophotometer, and minute amounts of dissolved oxygen were sufficient to destroy the dialuric acid. An attempt was made to determine the absorption spectrum of dialuric acid in freshly boiled buffer, pH 7.4. It showed a maximum at 275 *mμ*, but the molecular extinction was only about 20 per cent of the true value. Therefore, attempts were made to find a reagent that would prevent the oxidation of dialuric acid. Dialuric acid in concentrated sulfuric acid was stable and dilution with water gave a spectrum with a maximum at 270 *mμ* ( $\epsilon = 2900$ ). In 1 per cent hydrochloric acid, dialuric acid was not stable, but

the addition of 100 moles of cysteine for each mole of dialuric acid stabilized the spectrum and gave a maximum at  $270\text{ m}\mu$  ( $\epsilon = 3200$ ). This value was nearly the same as that obtained in sulfuric acid without cysteine. With the aid of cysteine, the spectrum of dialuric acid was also obtained at pH 7.4. It showed a maximum at  $275\text{ m}\mu$  ( $\epsilon = 16,500$ ). At pH 3.2, cysteine, added to a dialuric acid solution, did not prevent the fall in extinction as

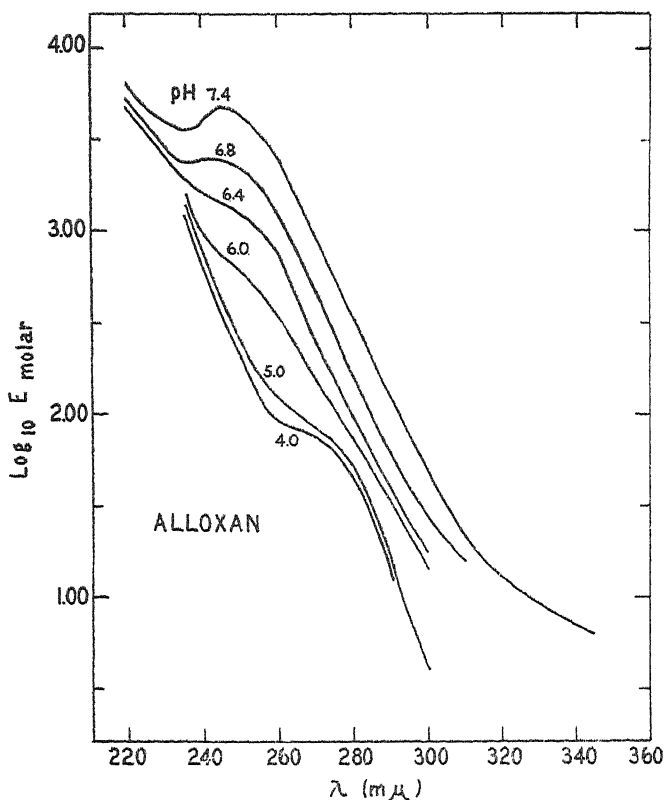


FIG. 2. Molecular extinction of alloxan at various pH values

well as at pH 7.4 or at less than pH 1.0. When a solution at pH 7.4 was diluted with buffer to give a final pH of 3.2, there was a maximum at  $275\text{ m}\mu$  ( $\epsilon = 9600$ )  $\frac{1}{2}$  minute after dilution which decreased after  $4\frac{1}{2}$  minutes to  $\epsilon = 7700$ . The spectra obtained with dialuric acid are shown in Fig. 3.

#### DISCUSSION

Alloxan, containing an active carbonyl as a result of its location between two carbamide groups, exists as the monohydrate. One of the hy-

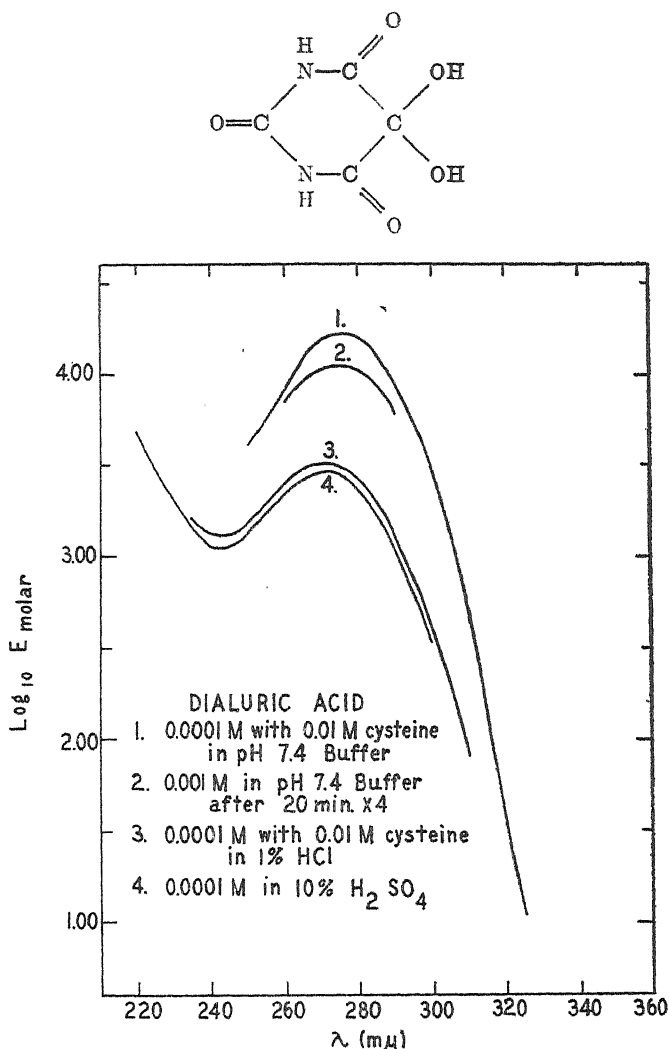


FIG. 3. Molecular extinction of dialuric acid

droxyl groups resulting from the hydration of the carbonyl is basic and will ionize in acid solution. It has a pK value of less than 1.0 (7). The hydrogen atoms attached to the 2 nitrogen atoms are acidic and will ionize in alkaline solutions. They have pK values of 7.2 and 10.0 (7, 12).

As a solution of alloxan is made progressively more alkaline, the dissociation of the first acidic hydrogen becomes more complete. At the same

time the half life of alloxan becomes shorter (Fig. 1); so that it would seem that the electronic structure necessary for decomposition is achieved by ionization of the first acidic hydrogen. At higher pH values at which both acidic hydrogens are ionized, the decomposition is much more rapid. With potentiometric methods, Richardson and Cannan (7) reported a half life of 1 minute at pH 7.4 and 30°. The value obtained by absorption spectra, of 0.9 minute at pH 7.4 and 37°, checks with their result.

Because of the instability of alloxan, stock solutions must be kept at less than pH 3.0, and experimental work at pH values greater than this must be interpreted with the instability of alloxan in mind.

With the dissociation of the first acidic hydrogen, the absorption spectrum of alloxan develops a maximum at 245  $m\mu$  which reaches an optimum value at pH 7.4. Increase of pH to 7.8 produces little change. That this maximum is related to ionization can be checked by calculation of the pK value from the absorption data and comparing it with that reported by other methods. This is accomplished (13) by proper substitution in the Henderson-Hasselbalch equation.

$$(1) \quad pK = pH + \log \frac{[HA]}{[A^-]}$$

For an acid,



The resultant or observed extinction is equal to the sum of the contribution of all absorbing substances.

$$(3) \quad E_{\text{total}} = E_{HA} + E_{A^-}$$

In turn each of these terms, by Beer's and Lambert's laws, is equal to its specific extinction multiplied by its concentration.

$$(4) \quad k([HA] + [A^-]) = k_{HA}[HA] + k_{A^-}[A^-]$$

Thus the value  $k$  is the specific extinction resulting in part from HA and part from  $A^-$ , the proportion depending on the pH. In alkaline solution, in which [HA] is at a minimum,  $k$  will tend to equal  $k_{A^-}$ , and in acid solution, in which  $[A^-]$  is at a minimum,  $k$  will tend to equal  $k_{HA}$ .

By solving equation (4),

$$(5) \quad \frac{[HA]}{[A^-]} = \frac{(k_{A^-}) - (k)}{(k) - (k_{HA})}$$

By substituting in equation (1),

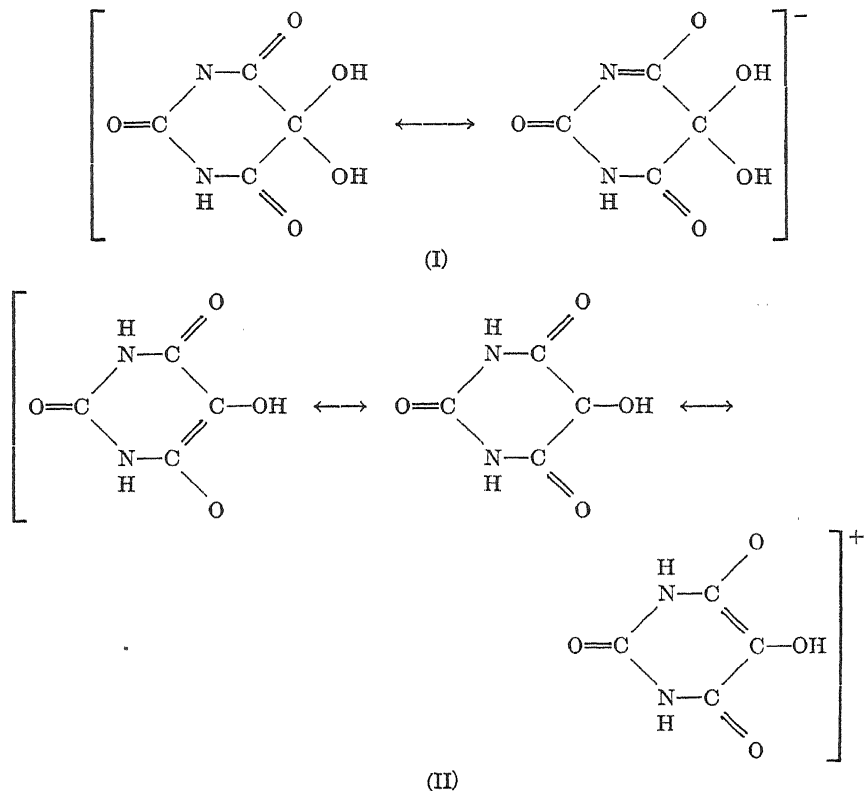
$$(6) \quad pK = pH + \log \frac{(k_{A^-}) - (k)}{(k) - (k_{HA})}$$



Since HA and A<sup>-</sup> have essentially the same molecular weight, pK can be calculated for any wave-length by substituting molecular extinctions ( $\epsilon$ ) for  $k$ ,  $k_{\text{HA}}$ , and  $k_{\text{A}^-}$ . At a given wave-length,  $\epsilon$  will be the value at the pH under consideration (pH 6.4 was used in calculation),  $\epsilon_{\text{HA}}$  will be the value in the most acid pH (4.0), and  $\epsilon_{\text{A}^-}$  the value in the most basic pH (7.4 or 7.8, which are nearly the same).

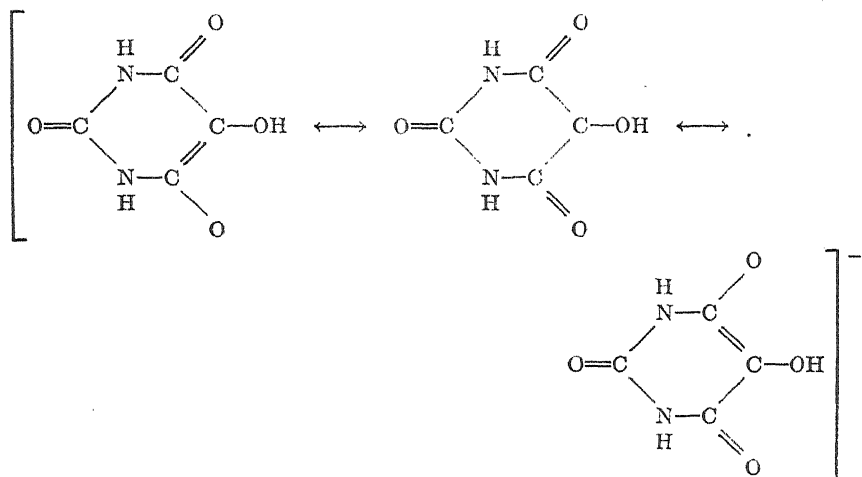
$$(7) \quad \text{pK} = \text{pH} + \log \frac{(\epsilon_{\text{A}^-}) - (\epsilon)}{(\epsilon) - (\epsilon_{\text{HA}})}$$

The calculated values are shown in Table I. The pK value averages about 6.8 and is only in rough agreement with the value 7.2 which is reported by others (7, 10). This may be accounted for by the fact that the values for  $\epsilon_{\text{A}^-}$  and  $\epsilon_{\text{HA}}$  are affected by the dissociation of other groups at more alkaline and more acid pH levels. This being taken into consideration, the absorption spectra correlate with ionization of the first acidic hydrogen of alloxan, and the structures (I) probably account for the maximum at 245 m $\mu$ .

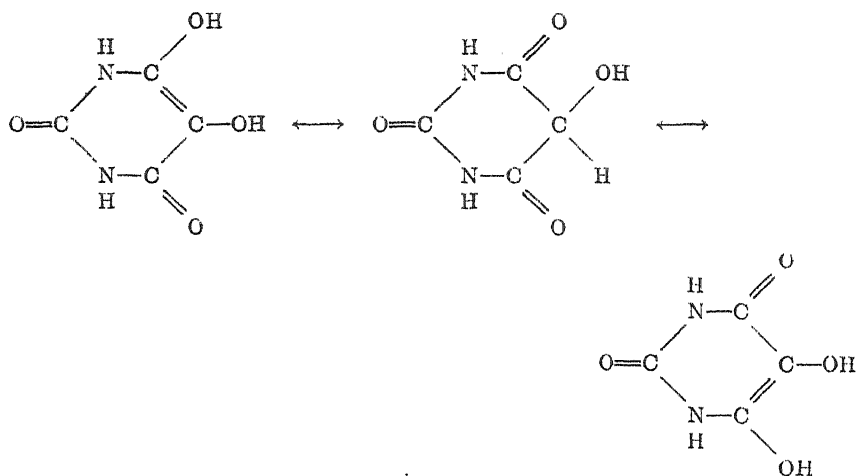


In acid solution the basic OH group of alloxan is expected to dissociate. In 10 per cent sulfuric acid there is a maximum at  $265\text{ m}\mu$  ( $\epsilon = 94$ ) and the extinctions in this part of the spectrum are higher than at pH 4.0. This supports the idea that the maximum seen in acid solutions results from the formation of the cation shown in structure II.

Dialuric acid is the reduction product of alloxan with one of the hydroxyl groups replaced by a hydrogen atom. This hydrogen is acidic and has a pK value of 2.8 (7, 12). Unlike alloxan the hydrogens attached to nitro-



(III)



(IV)

gens are not readily ionized; pK values are greater than 11.0. In acid solution, dialuric acid shows an ultraviolet absorption maximum at 270  $m\mu$  ( $\epsilon = 3200$ ). At pH 7.4 the maximum is increased in intensity and shifted to 275  $m\mu$  ( $\epsilon = 16,500$ ). This increase in intensity at pH 7.4 is consistent with ionization to the anion shown in structure III.

The fact that there is a maximum even in acid solution indicates that there may be an equilibrium (structure IV). This interpretation is consistent with that given for the absorption spectra of barbituric acid (3).

Calculation of a pK value for dialuric acid, in a manner similar to that used for alloxan, gives a value of 3.2 as compared with 2.8 reported in the literature. When the ease of oxidation of dialuric acid is taken into consideration, these values are considered as reasonable checks.

#### SUMMARY

1. The decomposition of alloxan was studied and shown to be a first order reaction with a half life of 0.9 minute at pH 7.4 and 37°. Rates at other pH values were also noted. At room temperature at pH 7.4 the half life of alloxan was 2.2 minutes.

2. The absorption spectrum of alloxan was determined at various pH values by extrapolation of the decomposition data at zero time. At pH 7.4 a maximum was found at 245  $m\mu$  ( $\epsilon = 4800$ ). This was interpreted as being the result of the ionization of the first acidic hydrogen. The pK value of this ionization was found to be 6.8. In 10 per cent sulfuric acid a maximum was found at 265  $m\mu$  ( $\epsilon = 94$ ), which was interpreted as being the result of the ionization of the basic OH group.

3. The absorption spectrum of dialuric acid was obtained by adding cysteine to prevent oxidation. It showed a maximum at 275  $m\mu$  ( $\epsilon = 16,500$ ) at pH 7.4 and at 270  $m\mu$  ( $\epsilon = 3200$ ) in 1 per cent hydrochloric acid. The maximum is thought to be the result of the keto-enol equilibrium and ionization of the acidic hydrogen on carbon 5. The pK value for ionization was found to be 3.2.

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# REACTIONS OF ALLOXAN AND DIALURIC ACID WITH THE SULFHYDRYL GROUP

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(Received for publication, July 19, 1948)

It was the purpose of this investigation to study the reaction between alloxan and the sulfhydryl group of cysteine and compounds containing cysteine.

Alloxan injected into animals produced diabetes (1). Cysteine or glutathione injected prior to alloxan, but not after alloxan, protected animals from diabetes (2). On the basis of these data it was postulated that alloxan produced diabetes by the inactivation of essential sulfhydryl enzymes. Since the action of alloxan was not affected by cysteine or glutathione given after the injection of alloxan, it seemed probable that inactivation would involve combination with an essential sulfhydryl group rather than oxidation to the disulfide linkage, since the latter would be a reversible change. Some workers (3, 4) believed that the main action of alloxan on sulfhydryl groups was oxidation. Therefore, more data on the reaction of alloxan with cysteine and its derivatives were desirable.

Since the compounds involved in this study were unstable, it was thought that the best approach would be to study the changes in the ultraviolet absorption spectrum of alloxan as it was mixed with cysteine and its derivatives.

## EXPERIMENTAL

All measurements were made on a Beckman photoelectric quartz spectrophotometer with 1 cm. quartz cells.

*Alloxan and Cysteine*—Alloxan (Eastman Kodak) was dissolved in acid solution (1 drop of concentrated hydrochloric acid to 100 cc. of water) to give a concentration of 0.05 M. Cysteine hydrochloride was made up as 0.05 M and 0.5 M solutions. Phosphate buffer (0.33 M) was made to give a final pH of 7.4 as checked with a glass electrode.

Mixtures of alloxan, cysteine, and buffer were made, and the extinction followed at 275 m $\mu$  until it became stable or started to decrease. At this

point the absorption spectrum was determined. The blank contained the same reagents as the sample except that alloxan was omitted. The results are shown in Fig. 1. Dialuric acid with cysteine under the same conditions gave similar results except that the extinctions between 320 and 360  $m\mu$  were a little lower.

*Alloxan and Glutathione*—Alloxan and glutathione solutions were mixed in a manner similar to that described for alloxan and cysteine. The ex-

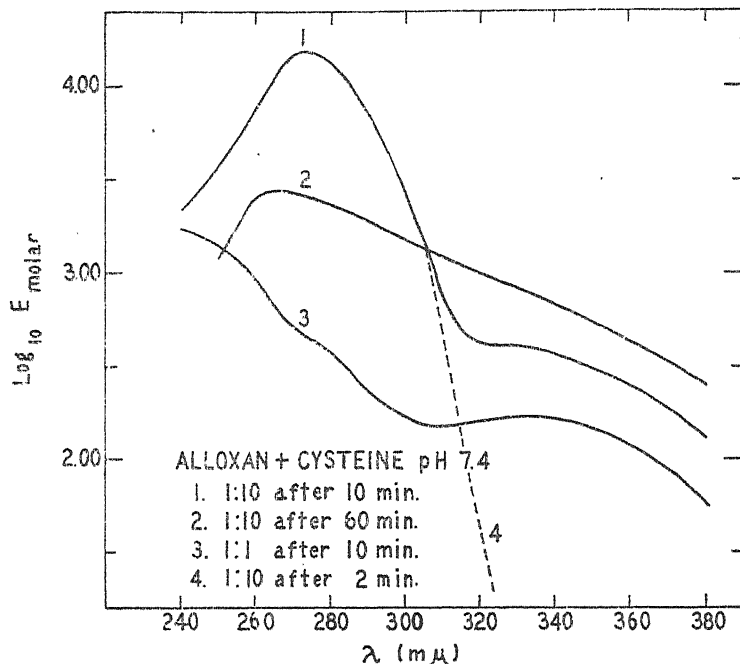


FIG. 1. Absorption spectra of cysteine with 0.0002 M alloxan (Curves 1 to 3) and 0.001 M alloxan (Curve 4).

inction was measured at half minute intervals at 305  $m\mu$  until the values were constant. The absorption spectrum was then measured, with use of a blank including all reagents except alloxan. When glutathione was in excess, the spectrum was also measured about 10 minutes after mixing. The results are shown in Fig. 2. Dialuric acid with glutathione gave essentially the same results as alloxan under the same conditions. The rate of formation of the maximum at 305  $m\mu$  with various concentrations of alloxan and glutathione is indicated in Table I.

Alloxan placed in buffer of pH 7.4 and followed at 305  $m\mu$  showed a drop

in extinction with decomposition. Oxidized glutathione added to alloxan at pH 7.4 did not affect the drop in extinction observed with alloxan alone.

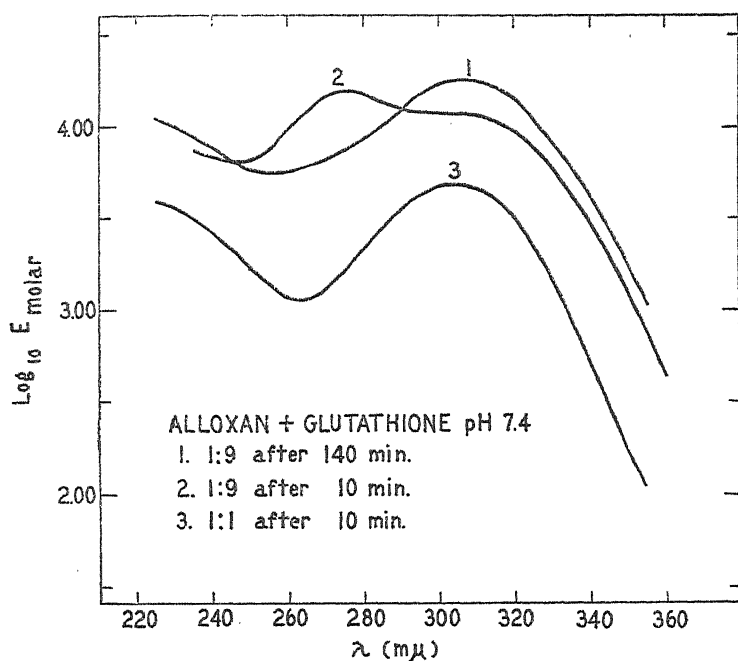


FIG. 2. Absorption spectra of alloxan (0.0002 M) and glutathione

TABLE I  
Rate of Formation of Substance 305

Molar concentration			$\epsilon_{\text{molar}}^*$ at 305 m $\mu$ , pH 7.4			
Gluta- thione	Alloxan	Cysteine	1 min.	2 min.	3 min.	Maximum
0.0001	0.001	0	5400	8200	9800	11,000 ( 6 min.)
0.0002	0.0002	0	1780	2850	3600	4,800 (14 " )
0.0022	0.0002	0	3900	5980	7240	17,400 (90 " )
0.0002	0.0002	0.0022	980	1300	1430	1,630 ( 6 " )
0.0001	0.001	0.010	310	350	310	350 ( 2 " )

\* Calculated on the basis of the substance in the lowest concentration.

*Alloxan and Protein*—Crystalline egg albumin<sup>1</sup> (5 cc. of 1.2 per cent) was mixed with alloxan (5 cc. of 0.01 M) and then added to buffer (pH 7.4) to

<sup>1</sup> We are indebted to Dr. K. C. Robbins for the sample of crystalline egg albumin.

make 25 cc. The extinction increased at 305  $m\mu$ , and the absorption spectrum was measured at 30 minutes. The blank contained identical reagents except that alloxan was omitted. A second solution was made which differed from the first in that the egg albumin was denatured by the incorporation of Duponol (5 cc. of 2.4 per cent). The results are shown in Fig. 3.

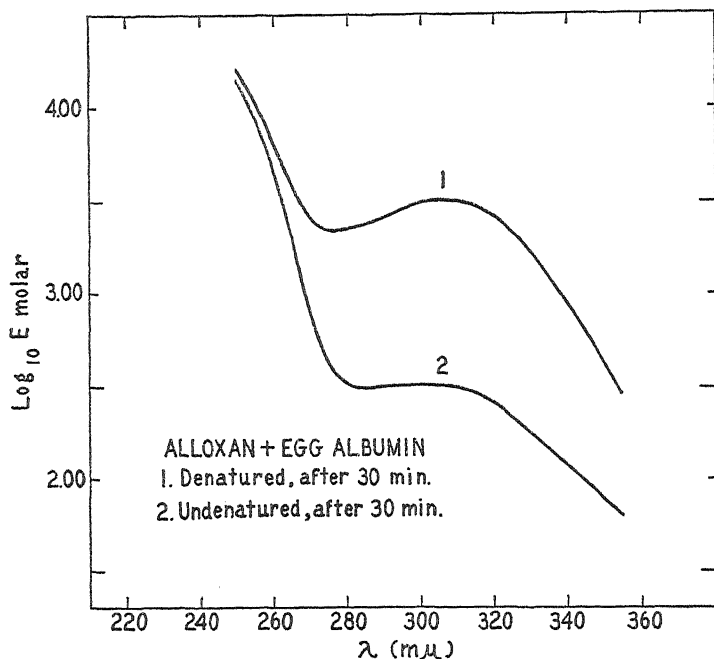


FIG. 3. Alloxan (0.002 M) with crystalline egg albumin (0.24 per cent) and Duponol PC (0.48 per cent) at pH 7.4 at 30 minutes (Curve 1), and without Duponol PC (Curve 2).

#### DISCUSSION

Alloxan on decomposition gives a spectrum which is much lower than that of alloxan itself (Fig. 4). A mixture of a sulfhydryl compound and alloxan, however, gives a spectrum which is higher than that of alloxan. Since the sulfhydryl compounds themselves have little ultraviolet absorption (5), and since they are present in the blank as well as the sample, the increase in extinction is indicative of a reaction between alloxan and a sulfhydryl compound. This reaction occurs at room temperature at pH 7.4.

The reaction of alloxan with 10 parts of cysteine (Fig. 1) gives a maximum at 275  $m\mu$  as the result of reduction to dialuric acid. With time the dialuric acid is oxidized by dissolved air, and a broad band appears between



320 and 360  $m\mu$ . This band is also noted when alloxan and cysteine are mixed in equal proportions. The substance causing this absorption is not known.

A mixture of 9 parts of glutathione and 1 part of alloxan gave an initial maximum at 275  $m\mu$  and strong inflection at 305  $m\mu$ . With time the maxi-

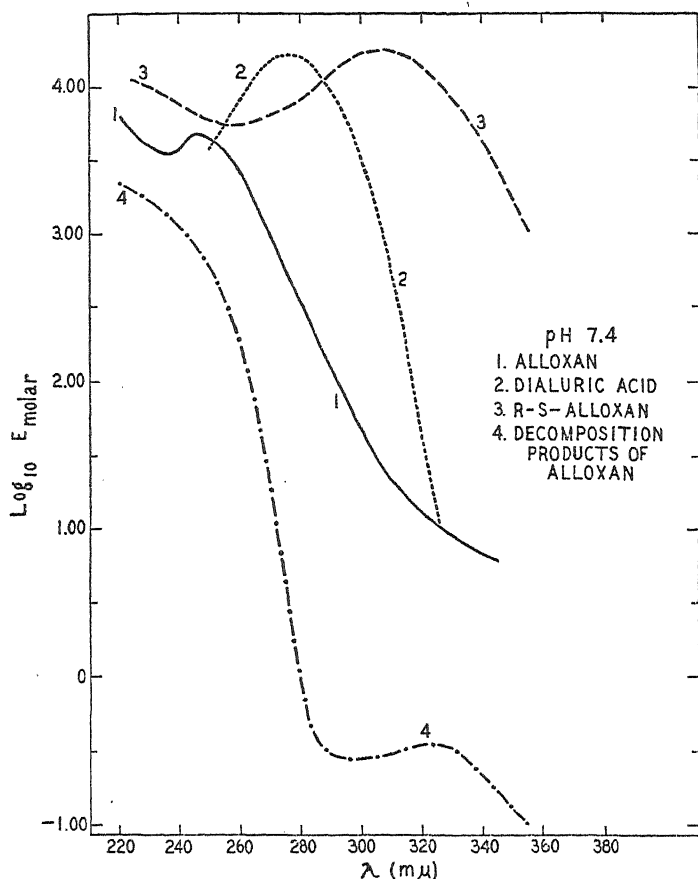


FIG. 4. Absorption spectra of alloxan and derivatives

mum at 275  $m\mu$  disappeared and the inflection at 305  $m\mu$  became a maximum ( $\epsilon = 17,400$ ) with an extinction greater than that of dialuric acid ( $\epsilon = 16,500$ ). A mixture of equimolar amounts of alloxan and glutathione gives a maximum at 305  $m\mu$ , but it is not possible to demonstrate a maximum at 275  $m\mu$  even if the spectrum is determined immediately after

mixing. That glutathione will reduce alloxan to dialuric acid is evident from the maximum at 275 m $\mu$ , which is seen early in the reaction of 1 part of alloxan with 9 parts of glutathione.

Dialuric acid is readily converted to alloxan by dissolved air (3, 6). Therefore it is not surprising that dialuric acid reacts with cysteine and glutathione to give essentially the same results as does alloxan. However, since cysteine and glutathione will reduce alloxan to dialuric acid, the question arises as to whether dialuric acid or alloxan is reacting to give substance "305." This question is answered by the results obtained with different ratios of alloxan and glutathione.

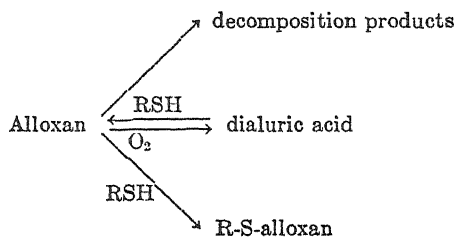
Reduction of alloxan to dialuric acid involves the reaction of 2 moles of glutathione for each mole of alloxan. Therefore, there should be the greatest amount of dialuric acid when glutathione is in excess, less when there are equal amounts of alloxan, and still less when alloxan is in excess. Under the last condition any small amount of dialuric acid formed is rapidly oxidized by air. Thus, the rate of reaction of alloxan with glutathione, if dialuric acid is an intermediate, should be greatest when glutathione is in excess, less when glutathione and alloxan are present in equal amounts, and least when alloxan is in excess. However, if alloxan is reacting directly with glutathione to form substance 305, then the rate of reaction should be least when alloxan and glutathione are present in equal amounts and greater when alloxan or glutathione is present in excess. The latter postulation fits the facts, as seen in Table I, and it is therefore possible to state that substance 305 is formed by the reaction of alloxan and glutathione. The greatest maximum is obtained with an excess of glutathione. When alloxan is in excess, decomposition is evident soon after the optimum extinction is reached. With excess glutathione some of the alloxan is converted to dialuric acid and slowly oxidized back to alloxan, which then can react with glutathione to form substance 305.

Alloxan mixed with 10 parts of cysteine is 60 per cent converted to dialuric acid in the 1st minute; therefore, the addition of cysteine to an alloxan-glutathione mixture should increase the rate of formation of substance 305 if dialuric acid is an intermediate. Since the results (Table I) are just the opposite, this is further indication of the fact that alloxan, rather than dialuric acid, reacts with glutathione to form substance 305.

Oxidized glutathione is formed from 2 molecules of the reduced form and has a disulfide linkage instead of a sulfhydryl group. Other functional groups are unchanged. Since alloxan does not react with oxidized glutathione to form substance 305, this substance must result from a reaction between alloxan and the sulfhydryl of glutathione. The exact nature of the product is unknown but it may be an addition product similar to that described by Schubert (7).

The sulfhydryl groups of native crystalline egg albumin will not give a test with nitroprusside reagent; however, after denaturation they are "free" and will give a test with nitroprusside (8). Some of these "free" sulfhydryl groups may be present in an amino acid configuration that will react with alloxan to form a substance with an absorption maximum at  $305\text{ m}\mu$ , whereas others may react with alloxan in a manner similar to cysteine and not contribute appreciably to a maximum at  $305\text{ m}\mu$ . Denatured protein does react with alloxan to produce a substance with a maximum at  $305\text{ m}\mu$  ( $\epsilon = 2800$ , based on the protein; molecular weight 34,000). The extinction is 10 times as great as that of the native protein with alloxan. The similarity of the curve to that obtained with alloxan and glutathione indicates that at least some of the sulfhydryl groups react in a manner similar to the sulfhydryl groups in glutathione. If substance 305 is an addition product of alloxan and glutathione, then the formation of an addition product between alloxan and a protein may be a mechanism for irreversible inactivation of essential sulfhydryl enzymes. The significance of this in the mechanism of alloxan diabetes was discussed in a preliminary note (9).

The reactions of alloxan with the sulfhydryl group are summarized in the accompanying diagram and the absorption spectra of the substances are illustrated in Fig. 4.



#### SUMMARY

Study of the evidence obtained from ultraviolet absorption spectra indicates the following: (1) Alloxan reacts with cysteine to form dialuric acid. (2) Alloxan reacts with glutathione to form dialuric acid and a substance giving a maximum at  $305\text{ m}\mu$  ( $\epsilon = 17,400$ ). This substance 305 results from a reaction involving alloxan and the sulfhydryl group of glutathione. It is thought to be an addition product of alloxan and glutathione. (3) Alloxan reacts with crystalline egg albumin to give a maximum at  $305\text{ m}\mu$ , which is increased 10-fold following denaturation of the egg albumin. (4) The substance giving a maximum at  $305\text{ m}\mu$  may be involved in the diabetogenic mechanisms of alloxan.

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# DETERMINATION OF TRYPSIN IN THE PRESENCE OF EGG WHITE TRYPSIN INHIBITOR AND DEMONSTRATION OF ABSENCE OF TRYPSIN FROM EGG WHITE

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(Received for publication, August 2, 1948)

Several investigators (1-4) have reported that egg white contains no active proteinase. On the other hand, Balls and Hoover (2) noted in 1940 that no method was available to determine whether an inhibited or inactive proteinase is present in egg white, which contains a large amount of trypsin inhibitor (ovomucoid (5)). A recent finding in this Laboratory has made it possible to determine trypsin in the presence of ovomucoid and therefore to demonstrate that, within experimental error, egg white contains no inhibited trypsin. In recent tests involving a number of proteinases, only trypsin was inhibited markedly by ovomucoid;<sup>1</sup> therefore it is also unlikely that inhibited proteinase of any kind occurs in egg white.

The question whether egg white contains proteinase originally became of interest when it was suggested many years ago that naturally occurring proteinase causes thinning of egg white when eggs are stored (1). The suggestion was given credence by the demonstration that addition of pancreatic proteinase to thick egg white accelerates thinning (2, 6). The possibility that naturally occurring proteinase causes thinning became less likely, however, when no active proteinase could be demonstrated in egg white; this possibility is now further minimized, since egg white contains no inhibited trypsin and therefore no traces of trypsin activity that might arise from incomplete inhibition.

The determination of trypsin in the presence of ovomucoid is based on the discovery that acetylated trypsin is active as a proteinase but is not inhibited by ovomucoid (7). As expected from this finding, the trypsin in a mixture of ovomucoid and trypsin or of egg white and trypsin became nearly fully active when either mixture was acetylated (Table I). Trypsin was not present in the precipitate that forms when egg white is dialyzed (Table I, Experiment 5).

\* Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

<sup>1</sup> The trypsin inhibitor is not a general proteinase inhibitor. Unpublished results of Lineweaver and Bean show that trypsin is more than 25 times as sensitive to the egg white trypsin inhibitor as is any of a number of other proteinases tested.

The absence of trypsin from egg white within the sensitivity limits of the experiments is shown by the data in Table II.

The possibility that inactivation of trypsin at the slightly alkaline pH (about 8) of egg white (where trypsin is known to be unstable) may have

TABLE I  
*Determination of Trypsin Content in Presence of Ovomucoid (Antitrypsin)*

Experiment No.	Material assayed	Trypsin activity, per cent of activity of control*	
		Original	After acetylation†
1	Trypsin, control	100	85
2	" 1 part, + ovomucoid, 15 parts	<1	70
3	" 1 " + egg white, 130 parts, equivalent to 15 parts ovomucoid	<1	65
4	Same as Experiment 3, but assayed by slightly different procedure		75
5	Insoluble fraction of egg white-trypsin mixture		<0.01

\* The crystalline trypsin obtained from Dr. Moses Kunitz was used in these experiments. The Anson ((8) p. 155) hemoglobin method of assay was used. The values for the acetylated samples were determined by interpolation from the curve relating color value and trypsin units for untreated trypsin, since the relation is practically the same for treated and untreated trypsin.

† Acetylation of the amino groups and isolation of the proteins by subsequent dialysis against dilute acid were carried out as described previously (7). Acetyl trypsin has about half the activity of crystalline trypsin. The figures exceed 50 per cent because the control loses activity more rapidly than acetyl trypsin during dialysis.

TABLE II  
*Absence of Trypsin from Egg White*

Experiment No.	Material assayed	Trypsin activity, per cent of activity of control*	
		Untreated	Acetylated*
1	Trypsin, control	100	85
2	Egg white from commercial eggs	<0.06	<0.006
3	" " cooled to 0° within 15 min. after egg was laid		<0.002

\* See foot-notes to Table I.

been responsible for our failure to find trypsin in commercial eggs was minimized by Experiment 3 of Table II. To reduce inactivation prior to acetylation, egg white was separated and cooled to 0° within 15 minutes after the eggs were laid. It was acetylated the next day and treated in the same way as the samples described in Table I.

Only speculation can be offered regarding the function of a specific trypsin inhibitor in egg white. The possibility of usefulness in the development and preservation of the egg immediately comes to mind; it is of more value, perhaps to compare this inhibitor with the trypsin inhibitor of the pancreas. The latter does play a very important part in regulation of the activation of trypsinogen and therefore in control of the action of trypsin ((8) pp. 97, 98). It is unlikely that a similar situation exists in egg white, since trypsin and probably trypsinogen do not occur therein. Our experiments do not prove unequivocally that trypsinogen is absent from egg white but do show that, if present, then acetyl trypsinogen (formed during the acetylation reaction) cannot be activated by acetyl trypsin. This conclusion follows from the findings that essentially the same trypsin activities were recovered in Experiments 3 and 4 of Table I, in which egg white containing added trypsin was acetylated and assayed, and in experiment 2, in which no egg white was present.

We are indebted to Dr. Moses Kunitz of the Rockefeller Institute for the crystalline trypsin used in this work.

#### SUMMARY

A mixture of trypsin and ovomucoid (the inhibitor of trypsin found in egg white) that exhibits no detectable proteolytic activity exhibits (when acetylated) at least three-fourths of the activity of acetyl trypsin, because acetylated trypsin is not inhibited by ovomucoid. Acetylation was used to show that egg white, which contains about 12 per cent of a trypsin inhibitor, contains less than 0.004 per cent trypsin (solids basis).

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# THE DISTRIBUTION IN NORMAL TISSUES OF RADIOACTIVE SULFUR FED AS LABELED METHIONINE\*

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(Received for publication, July 23, 1948)

The use of "methionine  $S^{35}$ " (1) for the study of liver metabolism has been the subject of several investigations recently. Thus far attempts to use this labeled amino acid in human experiments have not met with general approval because of uncertainties concerning the rate of turnover of sulfur in the body. It is considered essential that a reasonably accurate estimate of radiation dosage to various tissues be made experimentally before any extensive use of radioactive sulfur can safely be undertaken in clinical studies. An attempt at such an estimate is reported in this paper in which attention is paid only to the distribution of radioactivity in the tissues.

## Methods

*Experimental Animals*—Two adult male rats and a mongrel female dog were chosen for the distribution studies. The two rats were fasted for 36 hours and then each was fed approximately 1 gm. of methionine  $S^{35}$  mixed with meat paste. The dog was fasted for 48 hours and then given approximately 3.7 gm. of methionine  $S^{35}$  by stomach tube. This procedure caused vomiting. The material vomited was readministered.

To determine the "effective life" of  $S^{35}$  administered as methionine, two series of rats were used. The first series consisted of seventeen adult female rats. 200 mg. of methionine  $S^{35}$  were administered intraperitoneally in distilled water. The second series consisted of ten young male rats to which 8 mg. of methionine  $S^{35}$  were administered by stomach tube.

The animals were housed in individual cages with raised screen bottoms. The stock diet of Dickinson's dog pellets had the approximate composition, 24 per cent crude protein, 4 per cent fat, and 3.5 per cent fiber.

The methionine administered to all but the series of young male rats had

\* Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation, and in part from funds received from the United States Public Health Service. This study is a part of a joint investigation coordinated through the Josiah Macy, Jr., Foundation of New York. Other members of the group include Dr. Paul György, University of Pennsylvania; Dr. Sidney Madden, Emory University; and Dr. Lawrence Kinsell, University of California.

an activity of 91 microcuries per gm. In the latter series methionine with an activity of 2.24 millicuries per gm. was given.

*Selection and Treatment of Tissues*—All of the animals were anesthetized with ether, exsanguinated by cardiac puncture, and the tissues removed were immediately weighed. Whenever possible the organs were taken *in toto*. The pancreatic tissue of a rat, however, is so diffuse that removal of the whole gland, in most cases, is impossible. Separation of the pancreatic tissue from extraneous material was facilitated by immersion in water. Under these conditions the glandular tissue has a dark pink color which permits it to be separated easily from the surrounding fat. Muscle was uniformly chosen from the right fore limb, care being taken to exclude connective tissue. The right humerus was used in all bone studies. No attempt was made to separate bone from bone marrow. The stomach and intestines were washed repeatedly (four to six times) with physiological saline solution to separate the contents from the tissue.

The samples were prepared by two separate procedures. In the distribution studies the activity of the tissues was sufficiently high, so that a simple acid digestion procedure was adequate. In the "effective life" study an alkaline oxidation procedure was used which resulted in more complete oxidation of extraneous material and therefore less non-sulfur residue.

Acid digestion was accomplished in the following manner: The weighed tissues were placed in flasks and digested by refluxing for 24 hours with concentrated nitric acid. The solutions were made up to a known volume from which 0.5 cc. aliquot portions were removed for assay. These aliquots were dried on watch crystals under an infra-red lamp and counted.

Recoveries, in which known amounts of methionine  $S^{35}$  were added to various tissues, digested, dried, and counted, were within the expected limits of error. Nitric acid digestion, however, was never complete. Organic residues, though small, always remained. Attempts to oxidize the tissue completely and thereby reduce residual weight were made by treatment with 30 per cent hydrogen peroxide, perchloric acid, bromide, phosphoric acid, and fuming nitric acid, alone or as mixtures. These reagents either failed to lower the weight of the residue or did not permit quantitative recovery of added methionine  $S^{35}$ .

The procedure used for alkaline digestion was that suggested by Bailey (2). The tissues were placed in an oven and dried at  $105^{\circ}$  overnight. Approximately 500 mg. portions of the dried tissue were accurately weighed and placed in iron crucibles. The tissue was hydrolyzed for 6 to 8 hours with 10 cc. of 10 per cent NaOH on a steam bath. After the hydrolysates were concentrated to approximately 1 cc., 4 gm. of anhydrous  $Na_2CO_3$  were added and the mixture dried at  $105^{\circ}$  overnight. 4 gm. of  $Na_2O_2$  were then added and the mixture slowly fused to a cherry-red color.

The solution of the melt was acidified with concentrated HCl to approximately pH 3, filtered, and made up to a known volume. An aliquot of the solution was pipetted into a glass centrifuge tube with a removable flat bottom and carrier sulfate was added.<sup>1</sup> The solution was then heated over a steam bath and the sulfate precipitated from the warm solution by the dropwise addition of 10 cc. of a 10 per cent solution of BaCl<sub>2</sub>. The samples were centrifuged at 3000 R.P.M. and the precipitate washed twice with distilled water. The supernatant fluid was removed by siphoning after each centrifugation. The sides of the centrifuge tube were carefully washed down a third time with a small quantity of 50 per cent alcohol solution. The bottom of the centrifuge tube was removed, the alcohol evaporated at room temperature, and the sample dried over Drierite in a vacuum desiccator.

*Measurement of Radioactivity*—All radioactivity measurements were made with a commercial bell-shaped, self-quenching Geiger-Müller tube (window thickness 2.6 mg. per sq. cm.). The counter tube was mounted within a 1.5 in. lead shield which reduced background count to approximately 30 counts per minute. The counter was standardized against a uranium standard, to take into account changes in counting efficiency, before each day's counting. Resolution time determinations were made by the method of Reid (3), and a correction for the radioactive decay was made.

Since the  $\beta$  emanations of S<sup>35</sup> have a maximum energy of only 0.17 m.e.v., correction for self-absorption had to be made to refer all radioactive measurements to a constant predetermined weight. This correction was determined by the construction of a standard self-absorption curve. Counts were converted to disintegrations by the use of a counter factor of 11.5. Radioactivity in microcuries was calculated on this basis.

No corrections were made for back-scattering. All samples were counted until 10,000 counts had been recorded and duplicates were found to agree within 3 per cent.

To obtain an indication of the tissue dosage received the distribution and concentration of S<sup>35</sup> as a function of time were determined. While the amount of the isotope administered to each animal was identical ( $3.5 \times 10.6$  c.p.m.), the total amount of methionine varied greatly. This variability was occasioned by the synthesis of labeled methionine of considerably higher specific activity which permitted the administration of a physiological dose of 8 mg. to each young male rat. Because of the amount (200 mg.) administered to the adult females, elimination of S<sup>35</sup> undoubtedly pro-

<sup>1</sup> We wish to acknowledge the help of Dr. H. L. Fisher of the United States Industrial Chemicals, Inc., of Stamford, Connecticut, who kindly synthesized the radioactive methionine from sulfur obtained at Oak Ridge, Tennessee.

ceeded at a faster rate. In both instances the mode of administration, by stomach tube and by intraperitoneal injection for the male and female

TABLE I  
*Tissue Dosage in Equivalent Roentgens per Gm.*

Tissue	Young male rats	Adult female rats	
	25 days after administration of $S^{35}$	25 days after administration of $S^{35}$	130 days after administration of $S^{35}$
Liver.....	4.80	3.53	5.11
Kidney.....	5.76	3.98	6.30
Muscle.....	3.26	1.53	3.73
Pancreas.....		1.97	2.56
Testes.....	2.24		
Bone.....	8.16		
Blood.....	10.80		

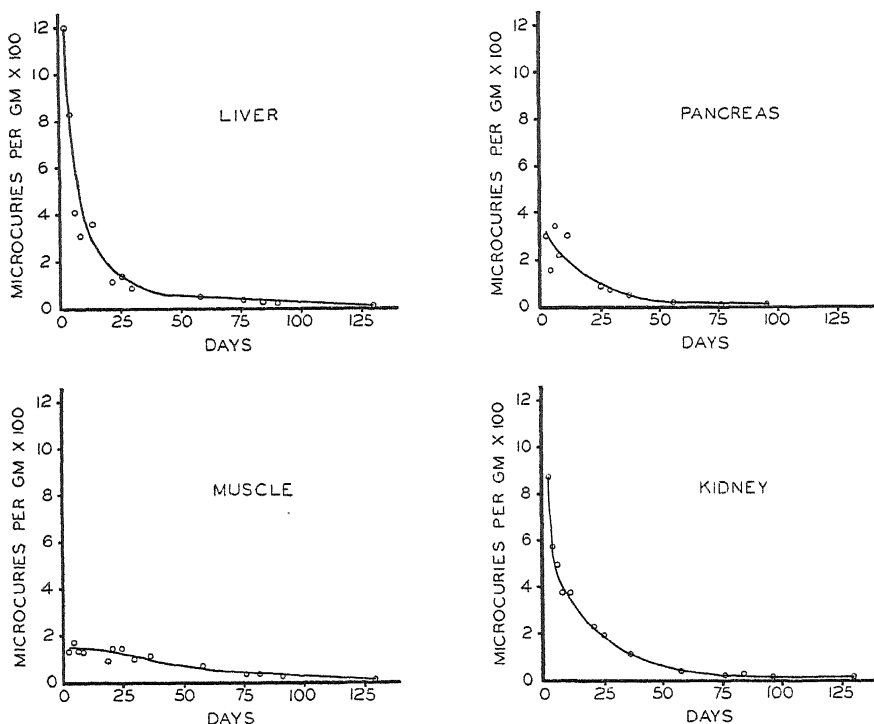


FIG. 1. The distribution of  $S^{35}$  in normal tissues of adult female rats

rats respectively, was chosen to approximate normal ingestion and still to permit quantitative dosage.

Since  $S^{35}$  emits only low energy  $\beta$ -rays, it is justifiable to assume that the dose of radiation received by the tissue is essentially confined to the regions containing the isotope. The total dose  $D$ , in equivalent roentgens, due to

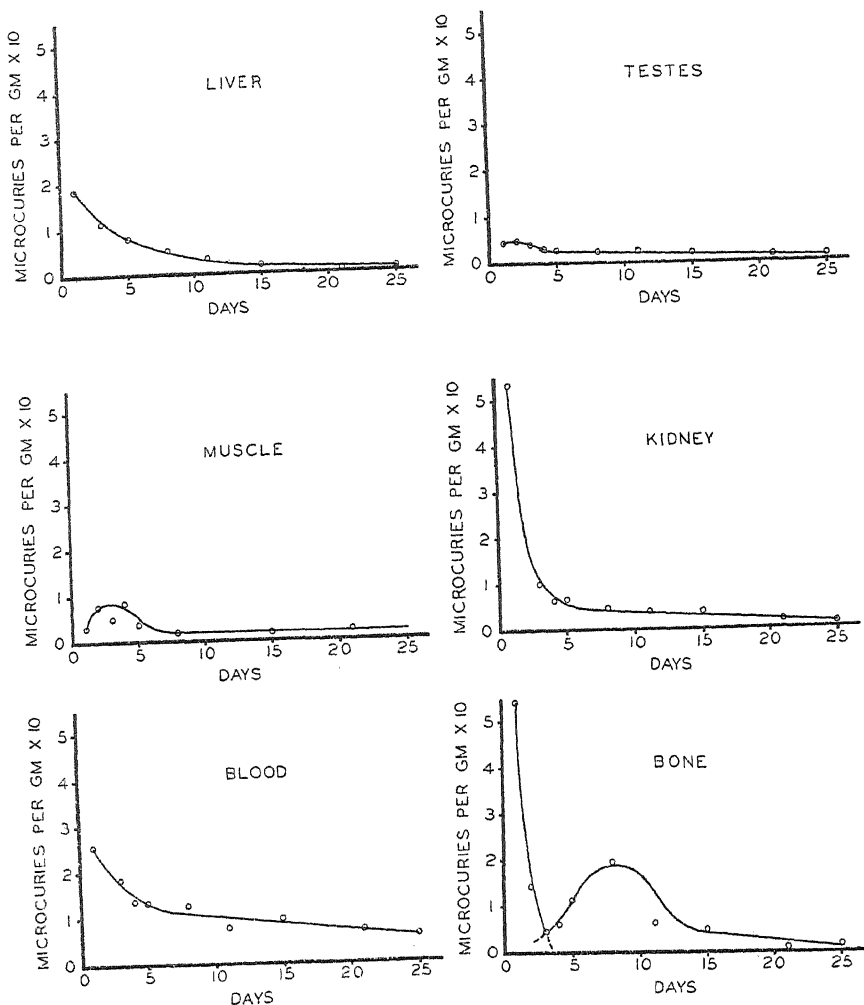


FIG. 2. The distribution of  $S^{35}$  in normal tissues of young growing male rats

complete disintegration of the  $S^{35}$  present in the tissue is  $D = 88\bar{E}TC$  equivalent roentgen per microcurie per gm. (4), where  $\bar{E} = 0.055$  m.e.v., the average energy per disintegration of  $S^{35}$ ,  $T$  = the half life of the isotope in days (87.1), and  $C$  = the concentration in microcuries per gm. of tissue.

Wherever biological synthesis, degradation, and elimination are occurring, the tissue concentration will be constantly changed by the turnover and elimination of activity from the tissue, and the half life ( $T$ ) is more accurately represented as the "effective life" ( $T'$ ) of the isotope in the tissue. Thus  $T'C$  can be represented by the area beneath the curves in Figs. 1 and 2. The values so calculated are presented in Table I.

TABLE II  
*Distribution Pattern of  $S^{35}$  in Rat*

Tissue	Rat I		Rat II	
	4 hrs. after administration		47 hrs. after administration	
	Tissue, wet weight	Wet tissue	Tissue, wet weight	Wet tissue
	gm.	counts per gm.	gm.	counts per gm.
Liver.....	7.960	38,860	9.870	29,664
Lung.....	1.740	14,014	1.542	31,145
Kidney.....	1.943	29,584	2.627	43,536
Adrenal.....	0.050	17,616		
Heart.....	0.817	10,827	0.940	24,695
Spleen.....	0.416	18,604	0.525	42,292
Pancreas.....	0.571	80,632	0.930	47,587
Stomach.....	2.351	52,492	1.900	34,171
Small intestine.....	6.145	5,089	4.967	45,760
Large ".....	1.712	21,257	2.097	25,975
Skeletal muscle.....	106.236*	4,959	132.568*	5,346
Testes.....	6.496	5,597	6.351	9,726
Brain.....			2.587	5,440
Blood.....	14.846*	8,414	18.526*	7,750
Stomach.....	2.370	199,970	2.50	3,154
Small intestine.....	2.490	93,315	4.70	38,347
Large ".....	4.700	5,790	2.40	34,667
Feces.....	2.400	825		

\* Calculated on the basis of body weight (5).

In so far as the metabolism of the human is comparable to that of the rat, this would be the dose received in equivalent roentgens per gm. of tissue from the oral administration of 18 microcuries of methionine  $S^{35}$  without extrapolation to a comparable weight basis.

#### DISCUSSION AND RESULTS

Tables II and III show the distribution patterns of  $S^{35}$  in the rat and dog, respectively. The most conspicuous finding is the lack of any selective localization of  $S^{35}$ . Following the oral administration of methionine,

radioactive sulfur is rapidly spread throughout the body. As might be expected, the initial high level of activity is found in the stomach contents 3 hours after ingestion of the labeled compound. Activity of the stomach contents declines rapidly over the following 44 hours due to movement through the gastrointestinal tract and absorption. Rapid absorption of methionine  $S^{35}$  is suggested by the high levels of activity in the gastrointestinal wall and the appearance of a measurable blood concentration.

TABLE III  
*Distribution Pattern of  $S^{35}$  in Dog*

Dog, 76 hrs. after administration		
Tissue	Wet weight	Wet weight of tissue
	gm.	counts per gm.
Liver biopsy.....	0.500	4,792 (29 hrs.)
".....	349.300	26,906
Lung.....	65.900	11,006
Kidney.....	43.220	15,213
Adrenal.....	0.307	25,235
Heart.....	57.110	2,593
Spleen.....	15.371	21,980
Pancreas.....	22.944	27,212
Stomach.....	81.800	55,384
Small intestine.....	239.500	19,439
Large ".....	46.700	2,454
Gallbladder.....	1.148	32,576
Urinary bladder.....	5.063	122
Rectum.....	9.860	17,246
Pituitary.....	0.005	
Nerve.....	0.346	5,715
Fat.....	0.242	2,576
Ovaries.....	0.328	
Aorta.....	2.866	10,533
Thyroid.....	0.720	4,466
Brain.....	76.200	2,792
Muscle.....	26.333	4,512

Rapid assimilation is indicated by the levels of activity of the pancreas, liver, and kidney as early as 4 hours after initial feeding. The concentration of activity in the gallbladder and gallbladder contents suggests, as Tarver and Schmidt have reported (6), that there is a rapid synthesis of taurine. A comparison of the distribution of  $S^{35}$  4 and 47 hours after administration demonstrates a general equilibration which results in a tendency for all tissues to approach a common level of activity. However, the skeletal muscle, testes, and brain of the rat and the fat, heart, and thy-

roid of the dog deviate markedly from this tendency at the end of 47 and 76 hours respectively.

#### SUMMARY

1. The distribution and concentration of  $S^{35}$  in the tissue of the rat and the dog were determined at intervals after the oral administration of methionine  $S^{35}$ .

2. Methionine was rapidly absorbed by the animals and  $S^{35}$  was present throughout the body within 4 hours.

3. No selective localization of activity was observed. The highest levels occurred in the intestine, pancreas, liver, kidney, and lung.

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# STUDIES IN VITRO ON METHEMOGLOBIN REDUCTION IN DOG ERYTHROCYTES

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(Received for publication, August 9, 1948)

The increase in red cell metabolism caused by methemoglobin (MHb) and the rôle of glucose as substrate in the reduction of MHb were observed by Warburg *et al.* (1). The reduction of MHb was recognized as an enzymatic process, but the precise mechanism has not been worked out. The work of Kiese (2) and more recently of Gutmann *et al.* (3) indicates that a yellow enzyme, methemoglobin reductase, and the pyridine nucleotides are involved. Gibson, giving additional evidence on the rôle of these enzymes, has shown that the failure of MHb reduction in congenital methemoglobinemia cases is associated with a low erythrocyte content of a flavin enzyme (4). Other substrates involved in the enzymatic reduction of MHb include lactic (5), malic, and fumaric acids (6, 7) and fructose (2). Studies dealing with the relative effectiveness of lactate, glucose, and fructose, and the inactivity of certain substrates in regard to MHb reduction have been reported by Kiese (2). The reduction is inhibited by malonate (6), iodoacetate, and fluoride (8), the effect of the latter being partially reversed by pyruvate (9). In the present communication more detailed results of findings reported previously (7) with several substrates and inhibitors are presented.

## Procedure

Heparinized blood obtained by venipuncture from six healthy dogs was used exclusively. MHb was formed by adding nitrite to the blood, and the reduction of MHb was followed at 38° in stoppered paraffined 25 ml. flasks, shaken gently. MHb and total hemoglobin were determined at intervals with a portable photometer as described by Andrews and Horecker (10).

Erythrocytes from the pooled blood samples of six dogs were washed by diluting with 8 volumes of sterile normal saline and centrifuging after 15 minutes at room temperature. After repeated washings, the cells were finally centrifuged at 1700*g* for 30 minutes and stored overnight in the refrigerator. In the morning, the packed cells were suspended in an equal volume of buffer which consisted of a mixture of equal parts of 0.85 per cent NaCl and 0.1 M Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4. The term "MHb cells" applies to erythrocytes washed five to seven times as described, after first having been incubated approximately 35 minutes at 38° with 0.07 ml. of 1 per cent

$\text{NaNO}_2$  per ml. of whole blood. In a given experiment a single batch of cells was used to prepare the flasks, and the dilution of cells and the time interval over which rates were measured were the same for all flasks. Usually rates were computed from the average of ten samples taken over a 5 to 7 hour period of incubation. When appreciable hemolysis occurred, the vessel was eliminated from further consideration.

### Results

*MHb Reduction in Fresh Whole Blood*—As shown by Cox and Wendel (11), the rate of MHb reduction is not constant but slows with decreasing MHb concentration. This is confirmed by the results in Table I which show that with decreasing levels of MHb the maximal reduction rate, com-

TABLE I  
*Relationship between MHb Level and Rate of Reduction in Whole Blood*

1 per cent $\text{NaNO}_2$ per 4 ml. fresh blood	Peak $C_{\text{MHb}}$	Maximal MHb reduction rate	
		$\frac{dC_{\text{MHb}}}{d \text{ time}}, K_0^*$	$\frac{d \log C_{\text{MHb}}}{d \text{ time}}, K_1^\dagger$
ml.	gm. per cent	gm. per cent per hr.	hr. <sup>-1</sup>
0.165	11.2	1.41	0.083
0.140	10.0	1.39	0.110
0.120	8.8	1.31	0.114
0.100	7.2	1.23	0.136
0.088	6.7	1.23	0.142
0.075	5.4	1.11	0.175

\* Derived from four or five points having the steepest slope.

† Represents the average of last four or five samples taken  $4\frac{1}{2}$  to 8 hours after addition of nitrite.

puted as the zero order reaction constant, decreases. Moreover, the rate is not linear if plotted as a first order reaction, but increases as the MHb level decreases. These constants are not generally applicable to whole blood but, as will be shown, may be applied to reduction with specific substrates. The zero order constant,  $K_0 = (dC_{\text{MHb}})/(d \text{ time})$ , represents the average decrease in gm. per cent of MHb per hour, and the first order constant,  $K_1 = (d \log C_{\text{MHb}})/(d \text{ time})$ , the average hourly decrease in the log of the MHb concentration.

Since with either expression the rate in whole blood is not linear, comparison can be made between rates only when the level of MHb is the same. Rates in fresh blood samples of six dogs determined under conditions which meet this requirement are shown in Table II. The average MHb peak for the 3 days was 7.7 to 8.0 gm. per cent for all the dogs. It will be noted that glucose does not increase the rate.

*MHb Reduction in Washed Erythrocytes*—Three washings were required to eliminate completely the reduction of MHb, although little activity was left after the first washing. The level of MHb attained after a given amount of nitrite increased 2 or 3 per cent each time the cells were washed from two to seven times, indicating progressive elimination of substances that react with nitrite.

MHb reduction with glucose proceeds at essentially the same rate whether the cells have been washed two or seven times. Thus, aliquots of a batch of cells washed an increasing number of times, from two through seven, packed uniformly, and treated with a standard amount of nitrite gave an average  $K_0$  value, from 2 to  $6\frac{1}{2}$  hours after adding nitrite, of  $0.96 \pm 0.02$  gm. per cent per hour for all washing régimes.

TABLE II

*MHb Reduction Rates in Freshly Drawn Dog Blood*

The flasks contain 2.0 ml. of cells + 2.0 ml. of homologous plasma + 0.10 ml. of 1 per cent  $\text{NaNO}_2$ . In Experiment 3 the flasks received 0.2 ml. of 5 per cent glucose.

Dog No.	Experiment 1. $K_1$ from $1\frac{1}{2}$ to 7 hrs. after $\text{NaNO}_2$	Experiment 2. $K_1$ from 2 to $7\frac{1}{2}$ hrs. after $\text{NaNO}_2$	Experiment 3. $K_1$ from $2\frac{1}{2}$ to $5\frac{1}{2}$ hrs. after $\text{NaNO}_2$
	<i>hr.<sup>-1</sup></i>	<i>hr.<sup>-1</sup></i>	<i>hr.<sup>-1</sup></i>
1	0.102	0.103	0.098
2	0.100	0.101	0.097
3	0.125	0.107	0.095
4	0.077	0.068	0.079
5	0.103	0.109	0.113
6	0.108	0.108	0.101

The fact that the MHb reduction rate with glucose is not altered by progressive washing indicates that coenzymes or other factors essential to the reaction are not dialyzed from the cell. This was confirmed by the failure of triphosphopyridine nucleotide (TPN) or diphosphopyridine nucleotide (DPN) to alter the rate of reduction in MHb cells supplemented with glucose. Negative results also were obtained with insulin, adrenal cortical extract, and epinephrine. Moreover, the addition of DPN was found to have no influence on the rate of MHb reduction with lactic or malic acid.

Of the many substrates which have been tested for ability to reduce methemoglobin by adding them in neutral solution to MHb cells, only the hexoses, D-glucose (Baker), D-fructose (Difco), D-mannose,<sup>1</sup> and D-galactose (Eastman), and three metabolic intermediates, sodium lactate (Mallinckrodt, 50 per cent), L-malic acid (Eastman), and fumaric acid (Eastman,

<sup>1</sup> Preparation of Dr. H. G. Fletcher, Chemistry Laboratory, National Institutes of Health; specific rotation,  $[\alpha]_D^{20} = +14.4^\circ$  ( $\text{H}_2\text{O}$ , equilibrium).

practical, recrystallized), have been found active. The observation that malic and fumaric acids show this activity was referred to in an earlier report (7). Kiese, in a paper which became available to us after our experi-

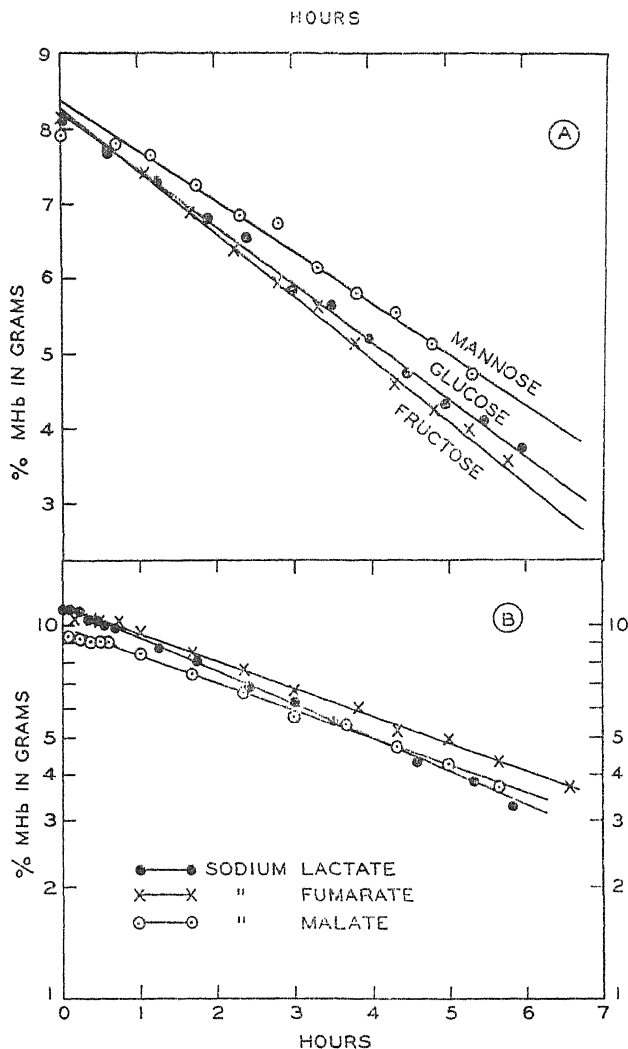


FIG. 1. MHb reduction with excess of substrates; cells suspended in saline-phosphate buffer. A, upper, linear plot; B, lower, log plot.

ments were completed, has also referred to this property of malate and fumarate (6). The MHb reduction curves have been plotted for each substrate, and it appears that linear regression is obtained on the log scale with

the intermediates and on the linear scale with the sugars (Fig. 1). Consequently, the first order constant,  $K_1$ , has been applied to rates produced by the three acids, and  $K_0$  to rates with the hexoses. The intermediates in optimal concentration bring about a more rapid drop in the MHB concentration than can be accomplished by the hexoses. Rates obtained one day are not accurately reproducible the next, but on a given day duplicate determinations check within 5 per cent of the mean.

The relative effectiveness of the intermediates in a wide range of concentrations is shown in Fig. 2. Comparison of the three acids was made at

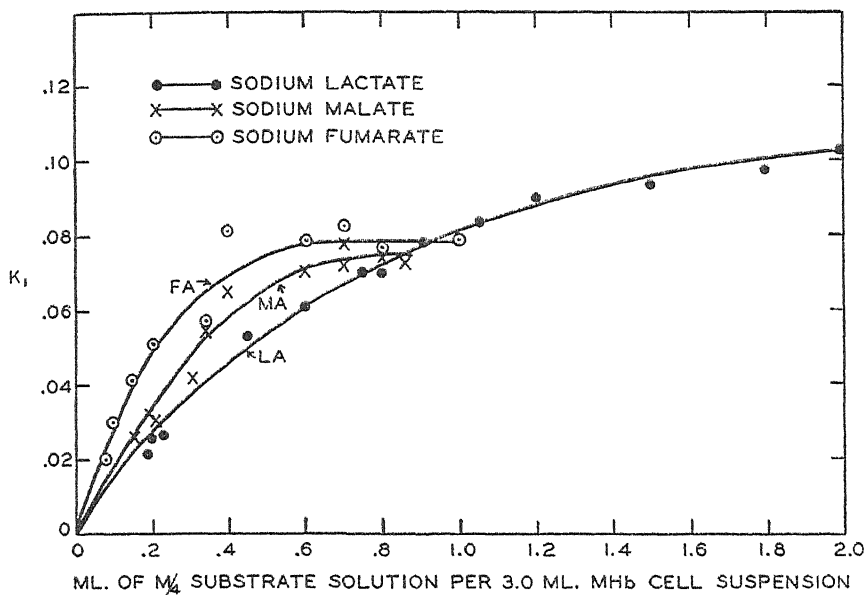


FIG. 2. Substrate concentration and MHB reduction rate

two to four levels on a single day, and the results obtained over several days were combined in Fig. 2. Although fumarate and, to a lesser extent, malate at all levels up to the optimal are more efficient than lactate, the latter when added in still higher concentrations, *i.e.* 1300 mg. per cent, produces a one-third faster rate. The maximal rate of reduction with lactate approximates that in whole blood.

With substrate concentrations smaller than those presented in Fig. 2, reduction does not proceed to completion. The total decrease in gm. of MHB per 100 ml. of cell suspension brought about by small doses within 5 hours is shown for the three intermediates and glucose in Table III. Here again fumarate is most and lactate least effective of the acids; but it is

obvious that glucose accomplishes more than lactate in twice the molar concentration. Identical results were obtained with 0.04 ml. of 0.25 M lactate solutions, whether prepared from 50 per cent sodium lactate or lithium lactate crystals.

A comparison of the methemoglobin-reducing activity of four hexoses is presented in Table IV. Galactose has the weakest effect. As regards the relative potency of the other sugars, the order of increasing activity is fructose, mannose, glucose, at low concentrations, and mannose, glucose, and fructose at high concentrations. Glucose reaches peak effectiveness between 35 and 60 mg. per cent. This may be compared with Dische's

TABLE III

*Total Decrease in MHb during 4 Hours with Low Substrate Concentrations*

The flasks contain 1.5 ml. of MHb cells + 1.5 ml. of saline-phosphate buffer + substrate.

Substrate			Experi- ment 1*	Experi- ment 2	Experi- ment 3
	ml.	M	gm. per cent	gm. per cent	gm. per cent
Sodium lactate	0.04	0.125	0.0		
	0.04	0.25		0.8	0.8
	0.05	0.25	0.6		
	0.08	0.25		1.0	1.8
" malate	0.04	0.25	1.0		1.2
	0.08	0.25			2.4
" fumarate	0.04	0.25	1.3		2.0
	0.08	0.25			3.8
Glucose	0.04	0.125	3.0	2.9	3.7

\* The MHb cells were not prepared exactly according to standard procedure this day in that the  $\text{NaNO}_2$  was added after the fifth wash and cells were then washed two more times.

observation that the rate of phosphorylation of hexose in erythrocytes is the same for glucose concentrations between 40 and 100 mg. per cent (12).

The disaccharide maltose (Difco and Pfanstiehl, c.p.), added to an MHb cell suspension at a level of 375 mg. per cent, gave an MHb reduction rate of 0.10, 0.11, and 0.14 gm. per cent per hour on different days. It seems unlikely that glucose contamination would be the explanation for the weak MHb-reducing action of maltose.

Some substrates were found to increase the rate of reduction in an additive manner. Table V shows results of typical experiments; it is seen that glucose is additive with lactate, malate, and fumarate, and lactate is additive with the latter two. In ten experiments the rate was 13 per cent greater on the average with glucose and lactate than with excess lactate alone. However, neither malate nor fumarate increases the rate in the

TABLE IV  
*MHb Reduction by Hexose*

The flasks contain 1.5 ml. of MHb cells in 1.5 ml. of saline-phosphate buffer + substrate.

Experiment No.	Substrate		$K_0$			
			Glucose	Fructose	Mannose	Galactose
	ml.	M	gm. per cent per hr.	gm. per cent per hr.	gm. per cent per hr.	gm. per cent per hr.
1	0.055	0.25				
	0.22	0.25	0.83	0.72	0.74	0.19
	0.11	0.5		0.93		0.13
	0.04	1.39			0.80	
	0.10	2.78		0.93	0.72	0.41
2	0.04	0.125		0.17	0.33	
	0.04	0.25		0.35	0.80	
	0.14	1.39		0.91	0.77	
3	0.023	0.125	0.38	0.10	0.33	
	0.045	0.125	0.66	0.23	0.57	
	0.075	0.25	0.85	0.53	0.69	

TABLE V

*Additive Effect of Metabolic Intermediates and Glucose on MHb Reduction*

The flasks contain 1.5 ml. of MHb cells + substrate + buffer to equalize the dilution.

Experiment No.	0.25 M substrate added				$K_1$
	Lactate	Malate	Fumarate	Glucose	
	ml.	ml.	ml.	ml.	hr. <sup>-1</sup>
1	1.8				0.092
	2.1				0.096
	1.8	0.4			0.095
	1.8		0.4		0.089
	1.8			0.4	0.106
2		0.71			0.079
		0.89			0.075
	0.26	0.71			0.090
		0.71	0.19		0.072
		0.71		0.23	0.090
3			0.7		0.076
	0.3		0.7		0.099
		0.3	0.7		0.076
			0.7	0.3	0.086

presence of excess of the other, and these substrates do not enhance the optimal reduction with lactate.

Similar experiments with the hexoses, typical of several which have been

carried out, are shown in Table VI. It is evident that glucose and fructose or glucose and mannose in combination give a rate about equal to that of glucose alone. Mannose, in combination with fructose, retards reduction by the latter to a rate at least as slow as that of mannose singly. Galactose, on the other hand, augments reduction by glucose and mannose and does not interfere with that by fructose.

TABLE VI

*Maximal Amount of Two Sugars Combined*

The flasks contain 1.5 ml. of MHb cells in 1.5 ml. of saline-phosphate buffer + the specified amount of 5 per cent solution of glucose, mannose, or galactose or 10 per cent solution of fructose + buffer to equalize the volumes.

Substrate	K <sub>0</sub>		
	Experiment 1	Experiment 2	Experiment 3*
	gm. per cent per hr.	gm. per cent per hr.	gm. per cent per hr.
0.3 ml. glucose	0.83	0.77	0.77
0.6 " "	0.78	0.81	0.76
0.3 " " + 0.3 ml. fructose	0.79	0.75	0.78
0.3 " " + 0.3 " "		0.78	0.80
0.3 " " + 0.3 " mannose	0.83	0.83	0.77
0.3 " " + 0.3 " "		0.79	
0.3 " " + 0.3 " galactose		0.89	0.85
0.3 " fructose	0.82	0.87	0.77
0.6 " "	0.93	0.83	0.82
0.3 " " + 0.3 " mannose	0.53	0.58	0.55
0.3 " " + 0.3 " "		0.49	
0.3 " " + 0.3 " galactose		0.85	0.86
0.3 " mannose	0.60	0.50	
0.6 " "	0.62		
0.3 " " + 0.3 " galactose		0.79	
0.3 " galactose		0.19	

\* 2 ml. of MHb cells + 2 ml. of saline-phosphate buffer in each flask on this day.

By taking samples from duplicate flasks at 3 to 5 minute intervals during the first 40 minutes and at half hour intervals thereafter, a comparison was made of the early phase of the MHb reduction curve with the later part. Table VII shows the slope of the line plotted for the early points to be somewhat less than that for the remainder of the curve.

The following substances added in large amounts have been found to have no effect on the MHb concentration in dog MHb cells: succinic, pyruvic, citric, acetic,  $\beta$ -hydroxybutyric, malonic, maleic, and glutamic acids, methyl glyoxal, acetaldehyde, D-ribose, D-xylose, D- and L-arabinose,  $\alpha$ - and  $\beta$ -galactose, L-sorbose, D-altrose, trehalose, melezitose, lactose, sucrose, and glucose-6-phosphate.



From the fact that glucose and lactic acid fail to reduce MHB in water solutions, it was concluded that the effect of these substrates depends on enzymatic mediators (13, 14). This is also the case with MHB reduction by malic and fumaric acids. On the other hand, malate and fumarate added in acid solution (pH 5.4) to hemolyzed red cells markedly accelerate MHB formation in comparison with equimolar addition of lactate at the same pH.

*Effect of Inorganic Ions*—Despite the fact that the erythrocyte is regarded impermeable to cations, the rôle of inorganic ions in MHB reduction might be in part elucidated by employing intact cells. To this end the rate of MHB reduction by glucose has been measured in solutions containing sodium cations alone or with potassium, calcium, or magnesium in

TABLE VII  
*Initial Delay in MHB Reduction with Hexoses*

The flasks contain 3.5 ml. of MHB cells + 3.5 ml. of saline-phosphate buffer + 0.55 ml. of 5 per cent glucose or mannose or 10 per cent fructose. The samples were taken at 3 to 5 minute intervals for the first 30 to 40 minutes and every 30 minutes thereafter. The rates were computed from the average of values in duplicate flasks.

Experiment No.	K <sub>0</sub>					
	Average for 1st 30 to 40 min.			Average from $\frac{1}{2}$ to 6 hrs.		
	Glucose	Fructose	Mannose	Glucose	Fructose	Mannose
	gm. per cent per hr.	gm. per cent per hr.	gm. per cent per hr.	gm. per cent per hr.	gm. per cent per hr.	gm. per cent per hr.
1	0.74	0.48	0.33		0.91	
2	0.50	0.45	0.48	0.88	0.94	0.74
3	0.57	0.40	0.38	0.75	0.80	0.69

concentrations comparable to those in Krebs-Ringer phosphate (15). In addition, the effect of adding phosphate to the simple saline medium has been determined for several substrates. Reference to Table VIII shows the marked accelerating effect of phosphate on MHB reduction by glucose, fructose, and mannose and the lack of effect on reduction by galactose. Other tests have shown that phosphate does not modify the rate of reduction by lactate or malate. Neither magnesium nor potassium added to a sodium chloride and phosphate medium appreciably alters the reaction rate, but with calcium a variable inhibition results.

*Inhibition*—The observation which first stimulated interest in the present subject involved failure of oxalated blood from nitrite-treated dogs to lose methemoglobin as expected. The rate of MHB reduction under conditions similar to those in Table I was then measured at two levels of oxalate, and the effect of added intermediates was determined. Table IX shows the partial inhibition in whole blood from oxalate at levels approximating those

required for anticoagulant action. Since reduction can be restored by adding metabolic intermediates, the inhibition is apparently due to impairment of glucose utilization. A somewhat similar effect has been obtained by suspending freshly drawn erythrocytes in an isotonic sodium citrate solution, or one slightly stronger but not hemolytic (Table X). Cells with glucose added show retarded reduction in citrate, whereas cells with no sub-

TABLE VIII  
*Rate of MHb Reduction ( $K_0$ ) As Influenced by Inorganic Ions*  
*A. Effect of Phosphate*

The flasks contain 2 ml. of MHb cells + 2 ml. of normal saline or saline-phosphate buffer + 0.3 ml. of 5 per cent glucose, mannose, or galactose or 10 per cent fructose.

Experiment No.	Medium	Glucose	Fructose	Mannose	Galactose
		<i>gm. per cent per hr.</i>	<i>gm. per cent per hr.</i>	<i>gm. per cent per hr.</i>	<i>gm. per cent per hr.</i>
4	Saline	0.52			
	" + phosphate	0.70			
5	"	0.40	0.43	0.37	
	" + phosphate	0.74, 0.72	0.81, 0.78	0.60, 0.65	
6	"				0.29*
	" + phosphate				0.28*

*B. Effect of Cations*

The flasks contain 2.0 ml. of MHb cells + 0.3 ml. of 5 per cent glucose + 2.0 ml. of buffer containing the ions designated in concentrations equal those in Krebs-Ringer phosphate buffer (14).

Experiment No.	$\text{Na}^+ + \text{PO}_4^{=}$	$\text{Na}^+ + \text{PO}_4^{=} + \text{K}^+$	$\text{Na}^+ + \text{PO}_4^{=} + \text{Mg}^{++}$	$\text{Na}^+ + \text{PO}_4^{=} + \text{Ca}^{++}$
	<i>gm. per cent per hr.</i>	<i>gm. per cent per hr.</i>	<i>gm. per cent per hr.</i>	<i>gm. per cent per hr.</i>
1	0.80			
	0.76	0.70	0.78	0.65
2	0.73	0.77	0.74	0.66
3	0.68	0.72	0.68	0.69
4	0.70	0.70	0.72	0.58

\* Value derived from averaging the rates in four flasks run simultaneously.

strate or with lactate added exhibit improved MHb reduction in citrate. Computing  $K_0$  for the rates gives the same result. The reason for this apparent acceleration is not clear but is probably not related to pH, for the citrate solutions had a pH of 7.35 and 7.42 (2.64 per cent) and that of the saline phosphate buffer was 7.30 with the glass electrode. It might be due to binding of an inhibitory cation such as calcium.

Iodoacetate was tested for inhibition of glucose-induced MHb reduction at the concentration of iodoacetate reported by Stannard (16) to inhibit

TABLE IX

*Oxalate Inhibition of Methemoglobin Reduction*

The flasks contain 3 ml. of fresh whole blood + 0.08 ml. of 1 per cent  $\text{NaNO}_2$  + oxalate + saline or 0.25 M substrate. The flasks for a given animal were run simultaneously and the rates computed over the same interval.

Dog No.	Oxalate concentration	Saline	$K_1$	Lactate	$K_1$	Malate	$K_1$	Fumarate	$K_1$
	mg. per ml.	ml.	hr. <sup>-1</sup>	ml.	hr. <sup>-1</sup>	ml.	hr. <sup>-1</sup>	ml.	hr. <sup>-1</sup>
6	3	0.8	0.044	0.8*	0.103				
2	3	1.0	0.031	0.3*	0.050			0.3	0.081
	3			0.6*	0.097				
	3			1.0*	0.077				
3, 4	3	0.6	0.024			0.3	0.091		
	3					0.6	0.091		
5	3	0.9	0.042	0.3	0.094	0.3	0.068†		
	3			0.6	0.095	0.6	0.071†		
	3			0.9	0.107	0.9	0.081†		
1	5	0.9	0.029	0.3	0.045				
	5			0.6	0.063				
	5			0.9	0.087				

\* 0.5 instead of 0.25 M.

† Some hemolysis occurred.

TABLE X

*Effect of Citrate on MHB Reduction in Fresh Erythrocytes*

Pooled blood obtained from six dogs on three occasions was centrifuged 30 minutes. Experiment 1, 0.053 ml. of 1 per cent  $\text{NaNO}_2$  added per ml. of cells before preparing cell suspension, and the rates measured for the interval of 2½ to 5½ hours after the start of incubation. Experiments 2 and 3, 0.100 ml. of 1 per cent  $\text{NaNO}_2$  added to flasks containing cell suspension and substrate, and the rates measured 1½ to 6 hours after nitrite addition. All flasks contain 2.0 ml. of cells + 2.0 ml. of buffer or citrate solution.

Medium	Experiment No.	No supplement	0.25 M substrate added			
		$K_1$	Glucose	$K_1$	Lactate	$K_1$
		hr. <sup>-1</sup>	ml.	hr. <sup>-1</sup>	ml.	hr. <sup>-1</sup>
Saline-phosphate buffer	1	0.022	0.3	0.103	0.6	0.088
	2	0.018	0.3	0.100	0.8	0.083
	3	0.026	0.3	0.118	0.8	0.097
2.28% sodium citrate	1	0.047	0.3	0.074	0.6	0.115
	2	0.026	0.3	0.075	0.8	0.100
	3	0.034	0.3	0.072	0.8	0.112
2.64% " "	1	0.035	0.3	0.066	0.6	0.118
	2	0.027	0.3	0.062	0.8	0.111

glycolysis but not the rate of oxidation of carbohydrates in tissues. It was not possible by this means to demonstrate a difference between the glucose MHB reduction path and the glycolytic path, for  $10^{-5}$  M iodoacetate caused 70 per cent inhibition of MHB reduction by glucose.

*Addition of Substrates to Whole Blood*—The failure of fluctuation in the glucose level between 40 and 400 mg. per cent to influence MHB reduction rates in dog blood *in vivo* or *in vitro* has been reported by Cox and Wendel, whereas Brooks observed acceleration of reduction in nitrite-injected rabbits after intravenous glucose (11). Kiese noted that in dogs intravenous lac-

TABLE XI

*Response of MHB Reduction Rates in Whole Blood to Added Substrates*

The flasks contain 3 ml. of fresh blood + the amount of 0.25 M substrate or saline and 1 per cent  $\text{NaNO}_2$  specified. The flasks on one line were run simultaneously and the rates computed over the same time interval.

Dog No.	$\text{NO}_2$ in each flask	Normal saline		Lactate		Malate		Fumarate	
		$K_1$		$K_1$		$K_1$		$K_1$	
	ml.	ml.	hr. <sup>-1</sup>	ml.	hr. <sup>-1</sup>	ml.	hr. <sup>-1</sup>	ml.	hr. <sup>-1</sup>
4	0.07	0.7	0.084	0.7*	0.095				
2	0.10	0.75	0.064	0.75*	0.074				
4	0.08	0.6	0.070	0.6	0.093	0.6	0.084	0.6	0.075
6	0.08	0.6	0.098	0.6	0.118	0.6	0.091	0.6	0.109
1, 2	0.08	0.6	0.098	0.6	0.111	0.6	0.100	0.6	0.098
5	0.08	0.6	0.101	0.6	0.131	0.6	0.101	0.6	0.111
4	0.10	1.0	0.031	1.0	0.063	1.0	0.044	1.0	0.042

\* 0.5 instead of 0.25 M.

tate protected against MHB (6). The increased MHB reduction rate in nitrited whole blood with lactate added may be seen in Table XI.

#### DISCUSSION

The negative effect with coenzyme on lactate and glucose activity in MHB cells and the similar rate of reduction by glucose, whether the cells are washed two or seven times, can be interpreted as indicating that either the coenzyme is not permeable or is not sufficiently dialyzed from the cell to make its concentration the limiting factor in determining the MHB reduction rate. That DPN is the limiting member in the MHB reaction with lactate and that the glucose reaction is mediated by TPN might be the best explanation of the ability of glucose to increase the MHB reduction rate in the presence of excess lactate. The increase in DPN (17) but not the increase in flavin-adenine dinucleotide (18) following incubation of unwashed human erythrocytes with niacin and riboflavin, respectively, has

been shown by Kohn and Klein to increase methylene blue-catalyzed oxidation of lactate in the red cell. DPN may then be limiting in this reaction.

The increased MHb reduction rate caused by glucose in the presence of excess lactate supports somewhat the contention that glucose reduction of MHb is not dependent on glycolysis. The greater effectiveness of glucose at low concentration than of lactate in double the molar strength concurs with the concept that glucose does not reduce MHb by merely splitting into 2 molecules of lactic acid.

Despite contradictory reports as to metabolism of the various sugars in blood (8, 12), it may be said that four hexoses are utilized for MHb reduction by dog erythrocytes. The specificity of the hexoses in this reaction might afford a means of identifying them under some conditions. The phosphate esters of glucose have long been recognized as impermeable (12), and adequate demonstration of this is afforded by the failure of glucose-6-phosphate to reduce MHb in intact erythrocytes. The finding that fructose and mannose together are less effective than fructose alone could be interpreted to be due to competition by the substrates for a single rate-limiting enzyme, the rate depending on the relative affinity of the substrates for the enzyme. The relative effectiveness of the hexoses at various levels could be explained if glucose, mannose, and fructose were all phosphorylated by hexokinase, fructose having the least affinity for the enzyme, and if isomerase activity limited the maximal utilization of glucose and mannose to a rate less than that of fructose. Galactose, which is additive with mannose and glucose, if not fructose, can be regarded as different from these other sugars in not sharing with them an enzyme limiting their reaction rates with MHb. This and the galactose independence of added phosphate suggest that the slow galactose metabolism, on the one hand, and glucose, fructose, and mannose utilization, on the other, occur by quite different pathways.

The significance of malate and fumarate utilization by erythrocytes is not clear when it is considered that the tricarboxylic acid cycle does not appear to exist in the erythrocyte. Whether, then, this reaction serves *in vivo* as a means of maintaining hemoglobin in the functional state or represents a vestigial non-functioning mechanism in the cells remains a question. Possibly, malate or fumarate occurs as a phase in the stepwise degradation of glucose to pyruvate. Stepwise degradation of glucose, originally proposed by Dickens (19), has been suggested by Gibson (4) as a probable route for glucose metabolism in methylene blue-treated erythrocytes.

#### SUMMARY

The reduction of MHb in dog erythrocytes by several substrates has been studied. At optimal levels the order of decreasing activity is lactate, fumarate and malate, fructose, glucose, mannose, and galactose. At low

levels the order of decreasing effectiveness for the metabolic intermediates is fumarate, malate, and lactate and for the hexoses is glucose, mannose, and fructose. Glucose at low concentration accomplishes more than the intermediates in double the molar strength. MHb reduction by the hexoses follows the pattern of a zero order reaction, whereas that with the intermediates resembles a first order reaction. Some of the substrates are additive with one another. Phosphate is necessary for a maximal response with glucose, fructose, or mannose, but not galactose. Oxalate and citrate inhibit MHb reduction by glucose; citrate, however, under some conditions enhances MHb reduction.

The authors express their appreciation to Dr. B. L. Horecker and Dr. H. D. Baernstein for valuable suggestions, and to these workers and Dr. C. S. Hudson and his coworkers for supplying certain of the materials tested.

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# A MICROMETHOD FOR THE DETERMINATION OF THE NUCLEIC ACIDS\*

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(Received for publication, August 16, 1948)

This paper describes a micro modification of the Schneider method (1) for determination of the nucleic acids in tissues. This modification has been applied successfully to 1 to 3 mg. (wet weight) samples of pollen mother cell material from plant anthers, the samples containing from 1.6 to 15.1  $\gamma$  of desoxyribose nucleic acid and from 8.6 to 58.0  $\gamma$  of ribose nucleic acid per mg. (wet weight).

The original method was devised for animal tissue; the present modification extends its usefulness to certain plant material and to tissues of which only very small samples are available.

Fig. 1 illustrates the micro equipment devised for the method. Samples are removed and placed on the bottom of the pestle of the micro Potter-Elvehjem homogenizer in a moist chamber to prevent evaporation of water prior to weighing on the micro balance. If it is possible to separate the tissue into discrete cells, as was the case in the plant material, a separate aliquot may be weighed for cell count, and the results of the analysis expressed per cell or per 10,000 cells, rather than per unit wet weight of tissue.

The samples weighed as above are homogenized for 1 minute with 0.03 ml. of cold distilled water, with a stirring motor revolving at 1500 R.P.M. Following this, the pestle is moved a short way up the tube, another 0.03 ml. of cold distilled water is added, and is sucked below the pestle by again moving it upward a short distance. Two 0.08 ml. portions of cold 10 per cent trichloroacetic acid are then added similarly and the washed pestle is removed from the tube. A stirring thorn is assigned to each tube at this point and is used to mix the tube contents at this and each subsequent washing. Fig. 2 illustrates a cold centrifuge block by means of which the micro tubes were centrifuged for 15 minute periods at 0-5° in a Sorvall model G angle head centrifuge without the use of a cold room.

The trichloroacetic acid-precipitated material is washed twice in the cold with 0.10 ml. portions of 10 per cent trichloroacetic acid and once in the cold with a 0.10 ml. portion of 5 per cent trichloroacetic acid prior to heat-

\* This research was carried out at the Brookhaven National Laboratory under the auspices of the Atomic Energy Commission.

ing. An extract pipette (Fig. 1) is used to remove the wash solutions from the sediment.

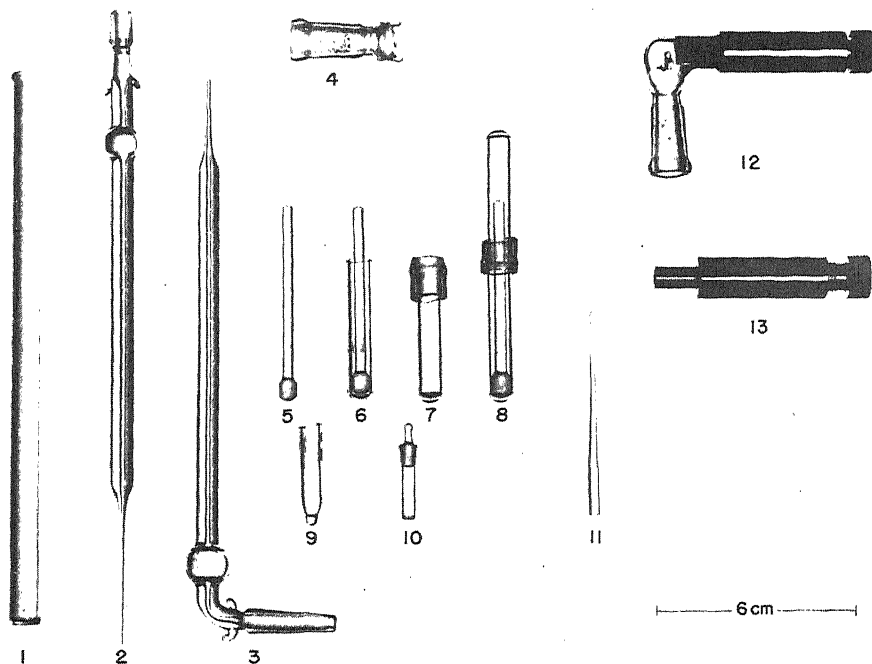


FIG. 1. Micro equipment. (1) Protective case for extract pipette. (2) Extract pipette, calibrated at 0.100 ml., standard taper 7/25 joint. (3) Aliquot pipette, calibrated at 0.033 ml. and at 0.100 ml., standard taper 7/25 joint. (4) Cap, used with aliquot pipette during immersion in boiling water. (5) Pestle of micro Potter-Elvehjem homogenizer. (6) Complete micro Potter-Elvehjem homogenizer. This is made by grinding to cylindrical form the sides of a ball of glass collected at the end of a glass rod, and grinding the pestle thus formed into the short length of tubing to be used as the homogenizer tube. The tube is then sealed off at one end and shaped to fit the bottom of the pestle by pushing the latter, coated with graphite, into the still soft bottom of the tube. (7) Closure for use during weighing. (8) Closure in place on a homogenizer, Tygon tubing used. (9) Storage vial. (10) Weighing vial for cell count aliquot. (11) Stirring thorn. (12) Completed suction device, standard taper 7/25 joint cemented on. (13) Metal suction device, Clay-Adams, Inc., New York.

Heating to release nucleic acids is carried out with a 0.05 ml. portion of 5 per cent trichloroacetic acid, and an additional 0.05 ml. portion is used to wash the sediment. The first extract is kept in the extract pipette, during the secondary washing of the sediment, by raising the pipette to a



nearly horizontal position. This is done by twisting the barrel of the suction device (Fig. 1) in the clamp which holds it during use. The second extract is drawn into the extract pipette after expelling a fraction of a drop of the first extract into the tube to rid the pipette tip of air; then additional 5 per cent trichloroacetic acid is drawn in immediately until the upper meniscus reaches the 0.100 ml. mark. The mixture is then expelled into a storage vial (Fig. 1) and made uniform by drawing it back into the extract pipette and expelling it several times (Fraction I).

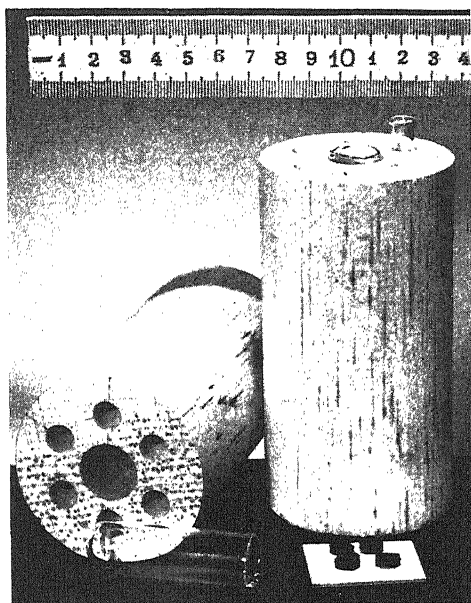


FIG. 2. Cold centrifuge block. The block is turned from pine wood. The central glass capsule is filled three-fourths full with crushed dry ice. The objects in the right foreground are rubber cushions.

With the plant material used, it was found necessary to wash the sediment with three additional 0.10 ml. portions of 5 per cent trichloroacetic acid to insure complete removal of ribose nucleic acid, although one additional 0.10 ml. portion sufficed for desoxyribose nucleic acid. These three additional washes were pooled as Fraction II and both types of nucleic acid were determined in Fractions I and II. As a check on the completeness of ribose nucleic acid extraction, the heating period was then repeated with 0.10 ml. of 5 per cent trichloroacetic acid and the resulting Fraction III was analyzed for ribose nucleic acid only. Only one of thirty-six samples yielded a significant amount of ribose nucleic acid in Fraction III.

Perhaps the additional washings will prove unnecessary when samples of animal tissue are undergoing analysis; this is suggested by the lesser number of extractions found necessary in the original Schneider method.

A 0.033 ml. aliquot is taken for the Dische diphenylamine reaction (1) in an aliquot pipette (Fig. 1). The reagent is then drawn in until the meniscus reaches the 0.100 ml. mark; then the column of liquid is drawn into the bulb of the pipette and mixed by extrusion again nearly to the pipette tip several times with the pipette in a nearly horizontal position. The capped (Fig. 1) pipette<sup>1</sup> is immersed except for a few inches of the capillary tip in a boiling water bath for the requisite time, being held in place by a wire mesh rack. It is withdrawn and inverted in a single motion after heating so that air being sucked into the pipette will not force liquid into the upper capillary. The pipette can then be brought to a nearly horizontal position for cooling of the bulb and the capped portion in a stream of running water.

The colored solution resulting is read in the Beckman spectrophotometer in the micro cuvettes<sup>2</sup> described by Lowry and Bessey (2). In such a cuvette having a 1 cm. light path, the extinction read, corrected for blank, multiplied by 12.8 gave micrograms of desoxyribose nucleic acid in the 0.6 to 10.3  $\gamma$  range.

A 5.0  $\gamma$  standard<sup>3</sup> gave single determinations on 11 separate days of 4.6, 5.0, 4.7, 5.1, 4.3, 4.5, 5.1, 5.0, 5.1, 5.1, and 4.3  $\gamma$ .

For the ribose nucleic acid determination, the aliquot pipette filled to the 0.033 ml. mark is emptied in 0.22 ml. of 5 per cent trichloroacetic acid in a 13 x 100 mm. tube and is washed out with the diluted solution. To the tube is then added 0.25 ml. of the orcinol reagent of Mejbaum (1) and the

<sup>1</sup> Dow-Corning Silicone stop-cock grease was used on the cap, and a rubber band was used to hold it in place.

<sup>2</sup> Obtained from the Pyrocell Manufacturing Company, 207 East 84th Street, New York.

<sup>3</sup> A convenient standard for use in the diphenylamine reaction for desoxyribose nucleic acid was prepared by heating about 18 mg. of thymonucleic acid (Schwarz Laboratories, New York 17) with about 15 ml. of 5 per cent trichloroacetic acid at 90° for 15 minutes, diluting to 25 ml., and filtering out the insoluble residue. This solution remained stable at refrigerator temperature for 5 months. However, it could not be used as a primary standard on the basis of its phosphorus content, since it gave only about two-thirds of the diphenylamine response per mg. of phosphorus given by a solution of desoxyribose nucleic acid in dilute alkali in which there was no residue of undissolved material. The permanent standard was therefore standardized by comparison with a temporary standard made by dissolving 18 mg. of thymonucleic acid (Dougherty Chemicals, 87-34 134th Street, Richmond Hill, New York) in a few ml. of water containing 1 drop 0.1 N NaOH and diluting to 25 ml. with water. The desoxyribose nucleic acid concentration in the temporary standard was calculated from a determination of its phosphorus content as recommended by Schneider (1).

color developed as usual and read in a micro cuvette. The extinction, corrected for blank, multiplied by 3.25 gave micrograms of ribose equivalent in the range 0.1 to 3.3  $\gamma$  of ribose. This figure was corrected for color contributed by desoxyribose nucleic acid by subtracting 0.023  $\gamma$  of ribose for each 1.0  $\gamma$  of desoxyribose nucleic acid present in the aliquot. This relationship was determined by experiment with the desoxyribose nucleic acid standard solution in the orcinol reaction. A 1.55  $\gamma$  ribose standard gave values in single determinations on 13 separate days of 1.68, 1.69, 1.50, 1.68, 1.64, 1.56, 1.51, 1.64, 1.65, 1.59, 1.58, 1.69, and 1.45  $\gamma$  of ribose. Since 1 mole of ribose nucleic acid gives the same color response as 2.6 moles of ribose rather than as 4 moles of ribose (1), micrograms of ribose nucleic acid are calculated from micrograms of ribose by multiplying by 3.3.

#### SUMMARY

A micro modification of the Schneider method for determination of the nucleic acids in tissues has been developed. This modification makes possible the analysis of tissue samples weighing 1 to 3 mg. (wet weight) and containing a total of 1 to 15  $\gamma$  of desoxyribose nucleic acid and 8 to 60  $\gamma$  of ribose nucleic acid.

The authors wish to thank Mr. Karl Walther of this laboratory for constructing the special glass equipment necessary for the performance of this method. They are also indebted to Dr. Arnold Sparrow of this laboratory, who suggested that the method be developed, and who furnished the plant anther material to which the method was applied.

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# METABOLISM OF OXALACETATE IN GLYCOLYZING TUMOR HOMOGENATES\*

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(Received for publication, August 31 1948)

In a recent report from this laboratory (1) it was shown that homogenates of several rat tumors showed negligible amounts of oxygen uptake when oxalacetate and pyruvate were used as substrates, in contrast to homogenates of brain, kidney, liver, and heart muscle. The oxidation in the latter tissues was dependent upon the presence of adenosine triphosphate (ATP). Kidney homogenates which had been incubated at 37° for 15 minutes could no longer oxidize oxalacetic acid (1) and, when added to fresh kidney homogenates, rapidly destroyed the ability of these to oxidize oxalacetic acid;<sup>1</sup> fresh homogenates of tumor behaved like the incubated kidney homogenates when added to the fresh kidney preparation.<sup>1</sup> In both cases the inactivation appears to be due to a preponderance of enzymes involved in the breakdown of the ATP system over the oxidative enzymes that maintain the ATP system.

Thus the results with tumor homogenates might be attributed to the failure of an inadequate oxidative mechanism to maintain the ATP system. It was desirable to study the ability of the tumor homogenate to oxidize oxalacetate under circumstances in which the ATP system could be maintained. Such a system was developed in this laboratory: glycolysis was very rapid in fortified tumor homogenates (2, 3) and was accompanied by the esterification of inorganic phosphate in good yield (3). It therefore is possible to study the ability of tumor homogenates to metabolize oxalacetic acid in the presence of an active glycolytic system, which is capable of maintaining the ATP system.

## EXPERIMENTAL

Oxalacetate metabolism was studied by analytical means, since it was not feasible to attempt to measure changes in O<sub>2</sub> tension in this system. Glycolysis was carried out by using the fluoride-blocked and pyruvate-supplemented system developed by LePage (3), under both aerobic and

\* This work was supported in part by grants from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council, and from the Jonathan Bowman Fund for Cancer Research.

<sup>1</sup> Unpublished experiments.

anaerobic conditions. Separate experiments were carried out with normal tissues to show that the oxidation of oxalacetate could be carried on in the presence of fluoride as employed in the glycolytic system (*cf.* (4)). The gas mixtures were prepared in the laboratory; in the case of the aerobic experiments, the gas was 95 per cent air and 5 per cent CO<sub>2</sub>. Gassing was accomplished by alternate evacuation and refilling (3, 5). The rate of CO<sub>2</sub> output was followed in the usual manner with the Warburg apparatus at 37.5° (3, 5).

The components of the basic reaction mixture were freshly prepared for each experiment so that each 2 ml. contained 0.1 ml. of 0.1 M K phosphate at pH 7.4, 0.45 ml. of 0.154 M KCl, 0.15 ml. of 0.5 M KHCO<sub>3</sub>, 0.3 ml. of 0.4 M nicotinamide, 0.1 ml. of 0.1 M glucose, 0.3 ml. of 0.01 M K ATP, 0.20 ml. of 0.2 per cent K DPN (diphosphopyridine nucleotide, approximately 85 per cent pure), 0.1 ml. of 0.1 M MgCl<sub>2</sub>, 0.15 ml. of 0.04 M K HDP (hexose diphosphate), and 0.15 ml. of 0.2 M KF. The glucose, nicotinamide, and bicarbonate were added in the solid form, with an appropriate amount of water. Each Warburg flask contained 2 ml. of this mixture, plus 0.3 ml. of a cold 10 per cent isotonic KCl homogenate of tumor tissue, 0.1 ml. of  $4 \times 10^{-4}$  M cytochrome *c*, and 0.4 ml. of 0.05 M K pyruvate *or* 0.4 ml. of freshly prepared 0.05 M K oxalacetate *or* 0.2 ml. of each. Finally, each flask contained 0.2 ml. of H<sub>2</sub>O or other addition to make the final volume 3.0 ml. Identical components were added to flasks in which the reactions were stopped for analysis after various time intervals, including zero time (*t*<sub>0</sub>), in order to follow the course of the reaction. Cold homogenate was added to cold flasks containing all other reactants, and the reaction was timed from the moment the flasks were placed in the 37° bath (3).

The reactions were stopped by adding 0.25 ml. of 65 per cent trichloroacetic acid from the side arm of each Warburg flask. The flask contents were then centrifuged in the cold and the supernatant fluid was analyzed for lactate (6), pyruvate, and oxalacetate as total keto acid (7) and inorganic phosphate (8) in every experiment, and in certain cases for citrate and malate. Citrate was determined by a modification of the pentabromacetone method (9, 10), while malate was determined by a new fluorometric method devised by Dr. J. F. Speck and kindly communicated to the authors before publication. Methods of estimating ATP phosphorus following barium fractionation and correction for hexose diphosphate were essentially as given by LePage in the manual by Umbreit *et al.* (5).

The tumors were all transplantable rat tumors (Flexner-Jobling carcinoma) taken 8 to 13 days after transplantation, while still non-necrotic.

### Results

The basic experiment can best be described in terms of a graph as shown in Fig. 1, which gives the typical results obtained when 0.4 ml. of either 0.05 M pyruvate or oxalacetate was added at  $t_0$ . In some six experiments of this type an additional group of flasks contained 0.2 ml. of pyruvate and 0.2 ml. of oxalacetate instead of 0.4 ml. of either pyruvate or oxalacetate, on the possibility that, in order to metabolize oxalacetate, the ATP of the

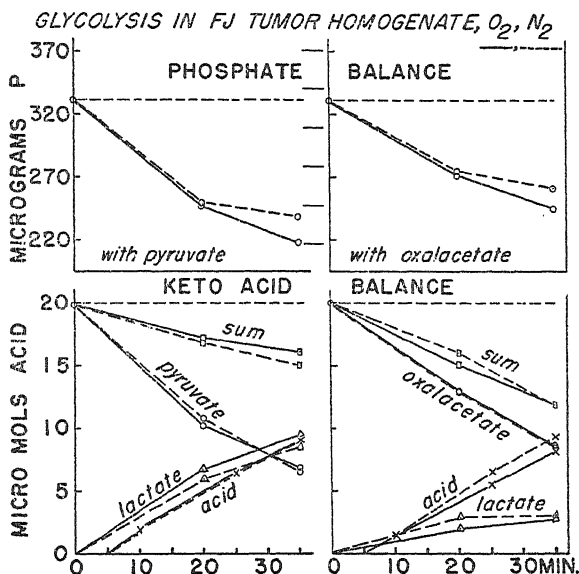


FIG. 1. Metabolism of pyruvate and oxalacetate in glycolyzing homogenates of Flexner-Jobling carcinoma in oxygen and in nitrogen. Each reaction mixture contained 30 mg. of fresh tissue (16 per cent dry weight). The upper part of the chart shows changes in inorganic phosphate concentration in micrograms per flask; the lower part shows the change in pyruvate, oxalacetate, and lactate concentrations in micromoles per flask. Curves marked "acid" are based on manometrically measured CO<sub>2</sub> output.

system would have to be maintained by a concomitant oxidation-reduction between triose phosphate and pyruvate. This did not prove to be the case, since oxalacetate proved capable of substituting for pyruvate in the coupled oxidation-reduction, as had been observed earlier in the case of muscle extracts (11). When a mixture of oxalacetate and pyruvate was used, the results were essentially an average of the results with either oxalacetate or pyruvate alone, and hence are omitted from Fig. 1.

The upper portion of Fig. 1 shows the results of the inorganic phosphate measurements, with each keto acid in the presence of oxygen or nitrogen. Phosphate uptake was vigorous in each of the four reaction conditions, and was consistently better in the case of pyruvate than with oxalacetate, as shown in Fig. 1. The mixtures taken off at  $t_{20}$  showed no difference in phosphate uptake in oxygen or nitrogen and the slight difference shown in Fig. 1 at  $t_{35}$  probably is not significant on the basis of similar experiments.

From the fact that the inorganic phosphate balance was favorable, *i.e.* inorganic phosphate disappeared throughout the course of the reaction, it would be inferred that the ATP reservoir was being maintained. However, proof of this was obtained by actual analysis following fractionation. The observed value for labile ATP phosphorus (corrected for HDP determined separately) was 142  $\gamma$  per flask in two flasks at  $t_0$ . In the six flasks that were stopped at  $t_{20}$  (see Fig. 1) the  $\Delta 7$  value averaged 49  $\gamma$  per flask, which represents the balance between the phosphate input from the oxidative step in glycolysis (none could come from phosphopyruvic acid since fluoride was present) and the phosphate output from ATP due to ATP-ase and hexokinase action.

The lower portion of Fig. 1 shows the results of the measurements of lactate,  $\text{CO}_2$  output, and keto acid disappearance. In each case, 20 micro-moles of keto acid were added. In the case of pyruvate (lower left, Fig. 1) the lactate formation determined chemically paralleled the acid formation as revealed by  $\text{CO}_2$  output, and was more than accounted for by the pyruvate disappearance. Fig. 1 also shows the results in terms of the sum of pyruvate plus lactate, which reveals that a small amount of pyruvate disappeared in a reaction other than direct reduction to lactate. Since pyruvate disappearance was the same in  $\text{O}_2$  or  $\text{N}_2$ , the reaction is assumed to be anaerobic and is possibly the dismutation to lactate plus acetate.

In the lower right portion of Fig. 1, the results obtained with oxalacetate are shown. In this case, the lactate formation did not parallel the acid formation, and much more oxalacetate disappeared than could be accounted for by lactate. The fact that the acid formation was about the same as in the case of the pyruvate addition indicated that triose phosphate was being oxidized to phosphoglyceric acid; in order for this to occur a hydrogen acceptor must have been present. Since the results were the same in oxygen and in nitrogen, and since the oxalacetate disappearing was unaccounted for, it would be inferred that oxalacetate was being reduced to malate, which might be dehydrated to fumarate, which in turn could be reduced to succinate. Attempts to demonstrate malate formation by polarimetric methods<sup>2</sup> were unsuccessful, due to the small amounts of malate encountered and due to the presence of interfering compounds.

<sup>2</sup> We are indebted to Dr. A. B. Pardes for the polarimetric measurements.



However, when Speck's method for malate was applied, it was possible to determine the malate formation. The analyses were carried out with 0.2 and 0.3 ml. of a 1:50 dilution of the trichloroacetic acid filtrates. There was a small interference which was due to the presence of glucose and hexose diphosphate (HDP), since filtrates from reaction mixtures that did not contain these substances gave absolutely no interference with the determination of added malate. Separate experiments showed that, when analyzing different aliquots of samples containing small amounts of glucose or HDP, one could extrapolate the values for malate to the "zero" aliquot to correct for the effect of the interference. The interference was appreci-

TABLE I

*Malate and Lactate Formation from Oxalacetate and Pyruvate during Aerobic Glycolysis, by Homogenized Flexner-Jobling Rat Carcinoma*

Conditions as in the text, except that glucose was reduced to 0.05 ml. of 0.1 M glucose per flask, and both the glucose and HDP were omitted from the  $t_0$  samples. Experiment otherwise comparable to right-hand portion of Fig. 1. Each flask contained 20 micromoles of added oxalacetate or pyruvate at zero time. The figures without parentheses represent flasks with oxalacetate; the figures in parentheses represent flasks with pyruvate.

Time	Oxalacetate (or pyruvate) per flask	Malate per flask	Lactate per flask	Sum, oxalacetate (or pyruvate) + malate + lactate	Inorganic phosphate per flask
<i>min.</i>	<i>micromoles</i>	<i>micromoles</i>	<i>micromoles</i>	<i>micromoles</i>	$\gamma$
0	20.0 (20.0)	0 (0)	0.3 (0.3)	20.3 (20.3)	333 (335)
15	13.2 (12.6)	3.6 (0)	0.9 (4.6)	17.7 (17.2)	327 (319)
15	13.0 (13.2)	3.8 (0)	0.9 (4.6)	17.7 (17.8)	317 (310)
30	7.9 (8.1)	5.5 (0)	2.4 (7.7)	15.8 (15.8)	306 (279)
30	8.1 (8.3)	5.4 (0)	2.4 (7.9)	15.9 (16.2)	293 (279)
45	5.1 (5.5)	6.4 (0)	3.5 (10.1)	15.0 (15.6)	287 (275)
45	5.4 (5.3)	6.4 (0)	3.3 (9.9)	15.1 (15.2)	288 (272)

able when 0.3 ml. of 0.1 M glucose per flask was employed and was slight when 0.1 ml. was used. The extrapolation to correct for the interference was unnecessary when the glucose addition was reduced to 0.05 ml. of 0.1 M glucose per flask, an amount very near the minimum level that could be used without affecting the glycolytic system (3). This amount of glucose was accordingly used in the experiment reported in Table I, in which the malate was determined on 0.2 and 0.3 ml. of the 1:50 dilution of the trichloroacetic filtrates. This experiment was comparable to that reported in Fig. 1, except that the conditions were aerobic throughout and the glucose was present in lower amount.

The fact that lactate was formed when oxalacetate was added (Table I) shows that oxalacetate was converted to pyruvate but the conversion was

not rapid enough to give a lactate production equal to that obtained with pyruvate; hence enough oxalacetate remained to compete with pyruvate for the hydrogen from the reduced DPN, and more malate than lactate was formed from the oxalacetate. It is of interest that no malate was formed from the pyruvate, hence no oxalacetate was formed from the pyruvate (Wood-Werkman reaction) under these conditions. The absence of malate from the  $t_0$  samples and from the incubated pyruvate samples is additional proof that the malate analyses on the samples that contained oxalacetate were valid.

Table I shows that the sum of lactate plus malate in the flasks that contained oxalacetate is equal to the lactate formed in the flasks that contained pyruvate. Moreover the sum of oxalacetate, lactate, and malate in the oxalacetate reaction mixtures at any moment was equal to the sum of pyruvate and lactate in the flasks to which pyruvate had been added. Thus the unaccounted for oxalacetate was equal to the unaccounted for pyruvate at any moment, and amounted to approximately 5 micromoles in 45 minutes. If this is calculated in terms of oxygen uptake equivalent to a dismutation reaction, the figure of 7.8 is obtained as a theoretical  $Q_{O_2}$ , which is very close to what has been observed in the case of slices of this tumor. The bulk of the unaccounted for oxalacetate may have been converted first to pyruvate, the formation of which is proved by the lactate figures, and may then have followed the pathway taken by the unaccounted for pyruvate. At any rate, attempts to demonstrate succinate formation in this type of experiment were unsuccessful. Determinations for citrate were also carried out, and, although there was a demonstrable difference between the experiments with pyruvate and with oxalacetate, the total amounts of citrate that accumulated were negligible in comparison with the keto acid that disappeared. Thus the 35 minute values for citrate from pyruvate, oxalacetate, or pyruvate plus oxalacetate in air were 0.04, 0.25, and 0.20 micromoles per flask in one experiment and 0.03, 0.15, and 0.19 in a replicate experiment, while the values in nitrogen were 0.025, 0.17, and 0.18.<sup>3</sup>

In an effort further to validate the results shown in Fig. 1, with respect to the  $O_2$  versus  $N_2$  data, a comparable experiment was set up by using  $N_2$  versus  $N_2$  in flasks to which a small stick of yellow phosphorus was added just before gassing (see (5)). The reactions were run for 35 minutes and stopped with trichloroacetic acid in the usual fashion. The phosphate values were erratic by 30 to 40  $\gamma$  in the case of the flasks that contained the yellow phosphorus, probably because of the slight fuming just before the oxygen was evacuated. The keto acid values and lactate values are given in Table II. The differences are not considered significant, and it is con-

<sup>3</sup> We are indebted to Mrs. G. G. Lyle for these determinations.

cluded that the unaccounted for pyruvate and oxalacetate were not disappearing by an oxidative pathway.

*Attempts to Fix Ammonia*—Since the tumor homogenates appeared to be unable to remove oxalacetate oxidatively, it was of interest to attempt to obtain oxalacetate removal by some other pathway. Mitchell and Houlihan (12) reported experiments with *Neurospora* in which "evidence is presented to indicate that the carbon chain of pyrimidines arises from oxalacetic acid." Since the incorporation of  $\text{NH}_3$  into oxalacetic acid might be expected to occur in the first steps of such a synthesis, with the loss of keto acid, the disappearance of oxalacetic acid in the reaction mixtures employed above was studied with and without  $\text{NH}_4\text{Cl}$  (0.0067 M final concentration), but no differences were observed. Thus, with 20 micromoles of oxalacetate

TABLE II

*Keto Acid Disappearance and Lactate Formation by Homogenates of Flezner-Jobling Tumors*

Anaerobic conditions,  $\text{N}_2$  versus  $\text{N}_2$  plus yellow phosphorus. Other conditions as in the text. Each figure is the average of two flasks. Incubation time, 35 minutes. The values are measured in micromoles.

Conditions	Pyruvate		Oxalacetate	
	Disappearance	Lactate formed	Disappearance	Lactate formed
$\text{N}_2$ .....	10.2	7.6	10.4	1.7
$\text{N}_2$ + yellow phosphorus.....	10.2	7.2	10.4	1.4

added the amounts utilized were 7.6 and 7.5 at 20 minutes and 12.4 and 12.4 at 35 minutes, with and without  $\text{NH}_4\text{Cl}$ . In other experiments,  $\alpha$ -ketoglutaric acid was substituted for oxalacetic acid and was tested with and without  $\text{NH}_4\text{Cl}$  in an attempt to form glutamic acid by reduction of  $\alpha$ -iminoglutaric acid with the hydrogen from the reduced DPN formed in glycolysis, since the glutamic system was shown to react with both DPN and TPN when a liver enzyme preparation was used (13). Neither  $\alpha$ -ketoglutaric acid nor  $\alpha$ -iminoglutaric acid (non-enzymatic formation in presence of  $\text{NH}_3$ ) was able to accept H from reduced DPN. In two experiments, the lactate formation was only 1.0 micromole at 35 minutes, compared to 10 micromoles in parallel tests in the presence of pyruvate. This is approximately the amount of lactate that can be formed in the fluoride system when no pyruvate is added (3). The disappearance of  $\alpha$ -ketoglutarate was very small with and without  $\text{NH}_4\text{Cl}$ , and there was an increase in inorganic phosphate (instead of the usual decrease as in Fig. 1) which was the same with and without  $\text{NH}_4\text{Cl}$ . These negative experiments are of course not conclusive since some essential condition for the reaction may have been omitted.

## DISCUSSION

The results in this report show that, even when the ATP system was maintained by the glycolytic mechanism, homogenates of Flexner-Jobling rat tumors were unable to oxidize oxalacetic acid. If this situation prevails in the tumors *in situ*, oxalacetic acid might be expected to be available for *alternative* paths of metabolism (14). The demonstration of an alternative pathway leading to a building block necessary for growth, *e.g.* uracil (12), would of course lend added significance to the finding that the oxidative pathway appears to be weak or missing. The failure to demonstrate such an alternative pathway in the present work is of little significance, since the correct conditions may be difficult to attain *in vitro*, and, even if attained, the rate may be very slow in comparison with the rates of the glycolytic reactions that have been reported here.

One may next consider whether oxalacetic acid is *formed* in appreciable amounts in tumor tissue. The failure to oxidize oxalacetic acid might seem to indicate that oxalacetic acid could not be formed in tumor tissue since the Krebs cycle of oxidations leads to oxalacetic acid formation as well as removal. But tumor tissues contain significant amounts of both succinic and malic dehydrogenases according to our earlier assays (15, 16),<sup>4</sup> and the substrates for these enzymes might reach the tumors via the blood stream or be formed in the tumor from amino acids. Oxalacetic acid might also be formed elsewhere in the body and transported to the tumors.

It would appear that both the problem of finding an alternative pathway for oxalacetate and the problem of whether oxalacetate is available in the tumors for such reactions might be studied *in situ* by means of C<sup>14</sup>-labeled precursors of oxalacetate.

Meanwhile other means of testing for the presence of the oxidative pathway need to be explored. It is desirable to know whether the tumor tissues contain negligible amounts of the enzyme or contain none of the enzyme, since proof of the latter condition would possibly be necessary if a somatic mutation were to be postulated as the cause of the irreversible loss of the enzyme. Proof that such a situation exists may not be readily obtainable because the admixture of small amounts of normal connective tissue and blood vessels with the tumor sample may not be readily eliminated.

## SUMMARY

The ability of fortified homogenates of Flexner-Jobling rat tumors to oxidize oxalacetate was studied. Since the oxidation in normal tissues occurs only under conditions that are compatible with ATP maintenance,

<sup>4</sup>The reduction of oxalacetate to malate reported in Table I is also proof that malic dehydrogenase is present in significant amounts.

the latter was accomplished in tumor homogenates by means of phosphorylating glycolysis, which proceeded actively in the presence of air. Oxalacetate and pyruvate disappearance was studied in a system in which pyruvate formation was prevented by fluoride. Pyruvate could be almost entirely accounted for as lactate, while oxalacetate was accounted for as a mixture of lactate and malate in air or in nitrogen. There was thus no evidence that significant amounts of oxalacetate could be oxidized.

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## DISTRIBUTION AND METABOLISM OF IODO- $\alpha$ -ESTRADIOL LABELED WITH RADIOACTIVE IODINE\*

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(Received for publication, May 3, 1948)

While the isolation of estrogens from gonads, adrenals, and placenta has indicated the sites of synthesis of these hormones (1, 2) and studies of urine have emphasized this pathway of excretion (3, 4), little is known of their fate in the body. Fragmentary data have been obtained by biological assay of blood (5-8), liver (5, 6, 9-11), bile (12-14), and other tissues (6, 10, 11). However, biological assays fail to demonstrate more than a small fraction of an injected dose of estrogens (15, 16) and, therefore, are not well suited for examination of the behavior of these compounds in the body. More recently, Berger has approached this problem by tracing radioactive bromotriphenylethylene in the organs and tissues of the mouse (17). In the present study  $\alpha$ -estradiol iodinated with radioactive iodine has been similarly investigated.

The only halogen derivatives of estrogens so far investigated from the chemical point of view have been the monobromo derivatives of estrone and estriol (18) and 2,4-dibromo- $\alpha$ -estradiol obtained by the action of N-bromoacetamide on free  $\alpha$ -estradiol (19). The iodinated derivatives used in the present experiments consisted at first of a crude mixture of mono- and diiodo- $\alpha$ -estradiol and later of crystalline diiodo- $\alpha$ -estradiol.

### *Chemical Experimentation*

*Preparation of Crude Radioiodo- $\alpha$ -estradiol*—Model experiments for the iodination of  $\alpha$ -estradiol were performed with either  $I_2$  in alkaline aqueous medium or N-iodoacetamide under anhydrous conditions. Both methods produced a yellow oil which, following chromatographic purification on alumina, and crystallization from benzene and petroleum ether, yielded a microcrystalline material whose melting point (173-174.5°, with decomposition) was depressed on admixture with pure  $\alpha$ -estradiol (m.p. 176-177°). Such crystals contained 480  $\gamma$  of iodine per mg. and were thus diiodo- $\alpha$ -estradiol (theoretical, 485  $\gamma$  of iodine per mg.).

For the preparation of the radioactive iodo- $\alpha$ -estradiol, the tracer  $I^{131}$ ,

\* Preliminary reports of this work have been presented at the Thirty-eighth meeting of the American Association for Cancer Research (Chicago, May, 1947) and at the Fourth International Cancer Research Congress (St. Louis, September, 1947).

present as iodide in 1 cc. of 1 per cent of NaOH, was mixed with 103.2  $\gamma$  of carrier potassium iodide and 26.6  $\gamma$  of potassium iodate and acidified with 0.2 cc. of 2.5 N sulfuric acid to release  $I_2$ . 100  $\gamma$  of  $\alpha$ -estradiol dissolved in 1 cc. of methanol and 0.2 cc. of concentrated ammonia were added. The reaction mixture (pH 9 to 10) was neutralized with glacial acetic acid, diluted to 10 volumes with distilled water, and extracted with three 15 cc. portions of freshly distilled ether. The combined ether extracts were washed with water twice and evaporated to dryness. The resulting oil was then taken up in 0.1 cc. of ethanol and diluted with 0.4 cc. of saline for injection.

*Composition of Crude Radioiodo- $\alpha$ -estradiol*—The nature of the injection material was determined by the use of the isotope dilution technique. For this purpose, the material was mixed with a large amount of non-radioactive iodo- $\alpha$ -estradiol prepared from 225 mg. of  $\alpha$ -estradiol treated for 24 hours at room temperature in absolute ethanol with 1 equivalent of N-iodoacetamide (155.7 mg. containing 106.6 mg. of I) prepared as described by Boismenu (20). After dilution with water and collection with ether the mixture was separated chromatographically on alumina. Three crystalline compounds and several oily fractions were obtained. Their iodine content and specific activity (amount of radioactivity per unit weight of iodine) were estimated (Table I).

Crystals 1 were shown by mixed melting point determinations and iodine content to be identical with the crystals of diiodo- $\alpha$ -estradiol isolated in the pilot experiments. Their purity was indicated by the constancy of their specific activity following recrystallization. Crystals 2 consisted of unchanged  $\alpha$ -estradiol. Crystals 3 were long, fine and needle-shaped, depressed the melting point of diiodo- $\alpha$ -estradiol, and contained an amount of iodine close to the theoretical for monoiodo- $\alpha$ -estradiol; namely, 319  $\gamma$  of I per mg. Their specific activity could not be measured because they were obtained in a small amount at a time when the radioactivity of the material was almost exhausted. It may be noted, however, that since Fraction 6, which was obtained after Crystals 3 in the chromatographic column, had a similar iodine content it may also be considered to consist of monoiodo- $\alpha$ -estradiol. Its specific activity, 5, was taken as representative of monoiodo- $\alpha$ -estradiol.

In order to calculate the content of each chromatographic fraction, it was assumed that only three substances were present; namely,  $\alpha$ -estradiol, monoiodo- $\alpha$ -estradiol, and diiodo- $\alpha$ -estradiol. For each fraction it was possible to set three equations, one based on the total weight, another based on the iodine content, and a third based on the specific activity. Thus, the three unknowns, namely the weights of  $\alpha$ -estradiol, monoiodo- $\alpha$ -estradiol, and diiodo- $\alpha$ -estradiol, could be calculated in each fraction.

A total of 102.5 mg. of monoiodo- $\alpha$ -estradiol and 64.2 mg. of diiodo- $\alpha$ -



estradiol was thus obtained. It is apparent that the small amount of crystalline moniodo- $\alpha$ -estradiol obtained (Crystals 3) is not due to a low yield but to difficulty in purification. The injection mixture consisted of  $\alpha$ -estradiol, moniodo- $\alpha$ -estradiol, and diiodo- $\alpha$ -estradiol in quantities roughly proportional to the totals listed at the bottom of Table I.

TABLE I  
*Chromatographic Analysis of Crude Iodo- $\alpha$ -estradiol*

	Eluate	Weight mg.	I content in $\gamma$ per mg. of fraction	Specific activity, counts per $\gamma$ I per min.	Calculated content in mg. of		
					$\alpha$ - Estra- diol	Mono- iodo- $\alpha$ -estra- diol	Di- iodo- $\alpha$ -estra- diol
Total oil used		340.6	260	3.7			
Fractions 1 and 2	Benzene	41.6	396	2	3.5	12.4	25.7
Crystals 1 (m.p. 173-174.5°, with decomposition)	" and benzene + 1% acetone	38.5	480	1.2	0	0	38.5
Crystals 2 (m.p. 176-177°)	Benzene + 4 and 8% acetone	48.2	0	0	48.2		
Fractions 3 and 4	Benzene + 50% acetone	12.5	340	4	1.3	11.2	0
Fraction 5	Benzene	4.9	50	5	4.2	0.7	0
Crystals 3 (m.p. 168-169.5°, with decomposition)	" + ether (50:1)	2.4	310		0	2.4	0
Fraction 6	Benzene + ether (50:1)	57.8	317	5	0	57.8	0
" 7	Benzene + ether (12:1 and 6:1)	21.9	245	4.5	5.2	16.7	0
" 8	Benzene + ether (3:1)	3.3	150	$\geq 5$	2	1.3	0
Total.....					64.4	102.5	64.2

*Preparation of Radioactive Diiodo- $\alpha$ -estradiol*—Pure diiodo- $\alpha$ -estradiol itself was used in a few animals to determine whether its distribution and excretion would differ from that of the crude material. 4 mg. of unlabeled, recrystallized diiodo- $\alpha$ -estradiol were dissolved in benzene together with a batch of crude radioiodo- $\alpha$ -estradiol. About 2.7 mg. of radioactive diiodo- $\alpha$ -estradiol were obtained by slow crystallization out of the mixture.

#### *Biological Experimentation*

In order to determine the biological activity of the iodinated compounds, both diiodo- $\alpha$ -estradiol and moniodo- $\alpha$ -estradiol were assayed in spayed

rats by the vaginal smear test, which is sensitive to 0.1  $\gamma$  of  $\alpha$ -estradiol. Neither of these substances had estrogenic potency at levels up to 100  $\gamma$ .

*Distribution of Labeled Material*—The distribution of the iodinated  $\alpha$ -estradiol was examined in about forty organs and tissues of seven diestrous female mice of the cancer-susceptible C3H strain and one diestrous female mouse of the cancer-resistant C57 strain. All of the animals were sacrificed 10 to 12 hours after a single subcutaneous injection.

Five of the animals were normal, non-tumor bearing animals. Animal *a*, a 4 month-old, 30 gm. C3H mouse, received 18  $\gamma$ , and Animal *b*, a 6 month-old, 26 gm. C3H mouse, received 15  $\gamma$  of crude radioiodo- $\alpha$ -estradiol in ethanol-saline in the dorsal region. Animal *f*, a 3 month-old, 11 gm. C3H mouse, received 2 mg. of radiodiiodo- $\alpha$ -estradiol in ethanol-saline in the right thigh. Animal *g*, a 3 month-old, 20 gm. C3H mouse, and Animal *h*, a 3.5 month-old, 20 gm. C57 mouse, received 0.54 mg. of radiodiiodo- $\alpha$ -estradiol in ethanol-saline and 1 per cent Duponol (sodium lauryl sulfate) in the right thigh.

Three of the cancer-susceptible animals bore mammary tumors, and, of these, two animals (*c* and *d*) were in good health and the third animal (*e*) seemed to be in poor condition. Animal *c*, a 9 month-old, 32 gm. mouse, received 3  $\gamma$ , Animal *d*, a 9 month-old, 26 gm. mouse, received 16  $\gamma$ , and Animal *e*, a 10 month-old, 25 gm. mouse, received 11.2  $\gamma$  of crude radioiodo- $\alpha$ -estradiol in ethanol-saline in the dorsal region. Animals *a*, *b*, *c*, *d*, and *e* were kept in individual mouse cages lined with filter paper for the collection of urine and feces. Animals *f*, *g*, and *h* were kept individually in specially constructed metabolism cages which provided a better separation of urine and feces.

At the time of autopsy, the animals were anesthetized with ether and blood was removed as completely as possible from the inferior vena cava. The organs were then removed, weighed, placed in test-tubes or flasks containing 1 cc. of 2 N NaOH for every 100 mg. of tissue weight, and heated in a water bath until homogenized. A 1 cc. aliquot was then pipetted onto a 40 mm. watch-glass and dried at 70° for 2 to 3 hours. The radioactivity was then counted under a bell-shaped  $\beta$ -ray Geiger counter tube. Urine and feces were treated in the same manner. In calculating the results, it was assumed that muscle comprises 50 per cent and blood 5 per cent of the body weight, half the blood being taken as plasma and half as cells. The results (Table II) showed a similar distribution of the crude iodo compounds (Animals *a* to *e*) and the pure diiodo- $\alpha$ -estradiol (Animals *f* to *h*).

Less than 1 per cent of the injected dose was recovered in the blood plasma, while a smaller but appreciable amount was found to have entered the red blood cells.

The bulk of the injected radioactivity was found in the various segments

TABLE II  
*Distribution of Radioiodo- $\alpha$ -estradiol*

	Crude iodo- $\alpha$ -estradiol in normal (non-tumor-bearing) mice			Crude iodo- $\alpha$ -estradiol in mammary tumor-bearing mice			Diiodo- $\alpha$ -estradiol in normal (non-tumor-bearing) mice		
	Animal	Per cent of injected dose	Concentration*	Animal	Per cent of injected dose	Concentration*	Animal	Per cent of injected dose	Concentration*
Blood									
Plasma	<i>a</i>	0.091	0.478	<i>c</i>	0.061	1.289	<i>f</i>	0.037	0.244
	<i>b</i>	0.115	0.615	<i>d</i>	0.100	0.276	<i>h</i>	0.166	2.825
				<i>e</i>	0.450	2.079			
Cells	<i>a</i>	0.036	0.188	<i>c</i>	0.024	0.377	<i>f</i>	0.015	0.090
	<i>b</i>	0.008	0.044	<i>d</i>	0.009	0.026	<i>h</i>	0.057	0.971
				<i>e</i>	0.091	0.422			
Gastrointestinal tract									
Stomach and contents	<i>a</i>	0.787		<i>c</i>	0.240		<i>f</i>	0.541	
	<i>b</i>	0.620		<i>d</i>	1.483		<i>g</i>	0.278	
				<i>e</i>	0.786		<i>h</i>	0.904	
Duodenum and contents	<i>a</i>	0.124		<i>c</i>	0.216		<i>f</i>	1.091	
	<i>b</i>	0.574		<i>d</i>	0.172		<i>g</i>	0.202	
				<i>e</i>	0.761		<i>h</i>	0.275	
Jejunioileum and contents	<i>a</i>	1.771		<i>c</i>	4.964		<i>f</i>	2.945	
	<i>b</i>	6.461		<i>d</i>	3.514		<i>g</i>	2.412	
				<i>e</i>	16.158		<i>h</i>	3.249	
Colon-cecum and contents	<i>a</i>	2.411		<i>c</i>	3.209		<i>f</i>	3.300	
	<i>b</i>	15.037		<i>d</i>	2.311		<i>g</i>	4.380	
				<i>e</i>	2.946		<i>h</i>	3.006	
Feces	<i>a</i>	30.419		<i>c</i>	72.457		<i>f</i>	21.995	
	<i>b</i>	17.590		<i>d</i>	30.456		<i>g</i>	30.765	
				<i>e</i>	2.125		<i>h</i>	24.271	
Glands related to gastrointestinal tract									
Liver	<i>a</i>	0.555	1.042	<i>c</i>	0.536	2.553	<i>f</i>	0.859	1.959
	<i>b</i>	0.754	1.956	<i>d</i>	0.861	0.867	<i>g</i>	0.570	4.063
				<i>e</i>	2.593	0.416	<i>h</i>	0.673	4.756
Pancreas	<i>a</i>	0.027	0.393	<i>c</i>	0.015	0.489	<i>f</i>	0.028	0.961
	<i>b</i>	0.020	0.420	<i>d</i>	0.037	0.227	<i>g</i>	0.012	0.863
				<i>e</i>	0.127	0.168	<i>h</i>	0.009	0.413
Gallbladder (with some bile)	<i>a</i>	0.002	1.709	<i>c</i>	0.004	18.298	<i>f</i>	0.599	21.594
	<i>b</i>	0.005	0.544	<i>d</i>	0.014	7.908			
				<i>e</i>	0.018	1.764			
Submaxillary	<i>a</i>	0.032	1.225	<i>c</i>	0.010	5.319	<i>f</i>	0.015	0.770
	<i>b</i>	0.069	2.835	<i>d</i>	0.011	0.249	<i>g</i>	0.013	1.379
				<i>e</i>	0.181	0.124	<i>h</i>	0.024	2.501

TABLE II—*Continued*

	Crude iodo- $\alpha$ -estradiol in normal (non-tumor-bearing) mice			Crude iodo- $\alpha$ -estradiol in mammary tumor-bearing mice			Diiodo- $\alpha$ -estradiol in normal (non-tumor-bearing) mice		
	Animal	Percent of injected dose	Concentration*	Animal	Percent of injected dose	Concentration*	Animal	Percent of injected dose	Concentration*
Glands related to gastrointestinal tract— <i>continued</i>									
Sublingual	<i>a</i>	0.001	0.177	<i>c</i>	<0.002	<0.318	<i>f</i>	0.010	0.568
	<i>b</i>	0.004	0.402	<i>d</i>	0.004	0.159	<i>g</i>	0.002	0.764
				<i>e</i>	0.006	0.712	<i>h</i>	0.003	1.447
Parotid	<i>a</i>	0.001	0.161	<i>c</i>	0.002	0.425	<i>f</i>	0.007	0.325
	<i>b</i>	0.010	0.561	<i>d</i>	0.006	0.223	<i>g</i>	0.005	0.788
				<i>e</i>	0.016	1.909	<i>h</i>	0.009	1.711
Respiratory system									
Lungs	<i>a</i>	0.015	0.380	<i>c</i>	0.017	1.085	<i>f</i>	0.013	0.220
	<i>b</i>	0.027	0.531	<i>d</i>	0.028	0.318	<i>g</i>	0.013	1.009
				<i>e</i>	0.095	0.153	<i>h</i>	0.024	1.673
Urinary system									
Kidneys	<i>a</i>	0.041	0.404	<i>c</i>	0.025	0.681	<i>f</i>	0.046	0.407
	<i>b</i>	0.052	0.562	<i>d</i>	0.061	0.282	<i>g</i>	0.031	0.899
				<i>e</i>	0.123	1.156	<i>h</i>	0.042	1.579
Bladder	<i>a</i>	0.007	0.729	<i>c</i>	0.025	0.681	<i>f</i>	0.004	0.445
	<i>b</i>	0.007	1.000	<i>d</i>	0.061	0.279	<i>g</i>	0.0008	0.107
				<i>e</i>	0.123	1.455	<i>h</i>	0.003	1.851
Urine	<i>a</i>	3.766		<i>c</i>	24.789		<i>f</i>	5.925	
	<i>b</i>	13.477		<i>d</i>	3.469		<i>g</i>	9.265	
				<i>e</i>	8.695		<i>h</i>	4.948	
Endocrine glands									
Thyroids	<i>a</i>	0.121	107.157	<i>c</i>	0.039	72.128	<i>f</i>	0.121	80.723
	<i>b</i>	0.145	91.162	<i>d</i>	0.289	163.340	<i>g</i>	0.026	115.284
							<i>h</i>	0.019	108.229
Adrenals	<i>a</i>	0.0003	0.244	<i>c</i>	<0.002	<2.553	<i>f</i>	<0.001	<0.465
	<i>b</i>	0.001	0.646	<i>d</i>	0.001	0.405	<i>g</i>	0.002	1.125
				<i>e</i>	0.003	1.277	<i>h</i>	0.001	1.481
Ovaries	<i>a</i>	0.0008	0.231	<i>c</i>	<0.002	<3.404	<i>f</i>	0.002	0.611
	<i>b</i>	0.002	0.544	<i>d</i>	0.002	0.286	<i>g</i>	<0.001	<0.830
				<i>e</i>	0.005	1.223	<i>h</i>	0.0006	0.705
Pituitary	<i>a</i>	0.0002	0.172	<i>c</i>	<0.002	<4.893	<i>f</i>	<0.001	<4.652
							<i>g</i>	<0.001	<2.296
							<i>h</i>	<0.0007	<2.865

TABLE II—*Continued*

	Crude iodo- $\alpha$ -estradiol in normal (non-tumor-bearing) mice			Crude iodo- $\alpha$ -estradiol in mammary tumor-bearing mice			Diiodo- $\alpha$ -estradiol in normal (non-tumor-bearing) mice		
	Animal	Per cent of injected dose	Concentration*	Animal	Per cent of injected dose	Concentration*	Animal	Per cent of injected dose	Concentration*
Lymphatic system									
Spleen	<i>a</i>	0.007	0.136	<i>c</i>	0.003	0.234	<i>f</i>	0.005	0.143
	<i>b</i>	0.018	0.266	<i>d</i>	0.032	0.191	<i>g</i>	0.004	0.406
Thymus				<i>e</i>	0.053	0.709	<i>h</i>	0.007	0.682
	<i>a</i>	0.0001	0.106	<i>c</i>	<0.002	<2.978	<i>f</i>	0.001	0.100
	<i>b</i>	0.003	0.412	<i>e</i>	0.002	0.911	<i>g</i>	0.001	0.346
							<i>h</i>	0.003	0.499
Lymph nodes	<i>a</i>	0.011	0.308	<i>c</i>	0.025	0.638	<i>f</i>	0.018	0.267
	<i>b</i>	0.016	0.434	<i>d</i>	0.016	0.190	<i>g</i>	0.012	1.331
							<i>h</i>	0.008	0.854
Accessory sex organs									
Uterus	<i>a</i>	0.020	0.345	<i>c</i>	0.002	0.596	<i>f</i>	<0.001	<0.237
	<i>b</i>	0.017	0.398	<i>d</i>	0.022	0.241	<i>g</i>	0.007	1.125
				<i>e</i>	0.019	1.273	<i>h</i>	0.011	1.186
Cervix	<i>a</i>	0.004	0.416	<i>c</i>	<0.002	<2.553	<i>g</i>	0.002	0.857
	<i>b</i>	0.006	0.792	<i>d</i>	0.002	0.183	<i>h</i>	0.001	0.854
				<i>e</i>	0.019	1.040			
Vagina	<i>a</i>	0.006	0.492	<i>c</i>	0.006	2.766	<i>f</i>	0.005	0.707
	<i>b</i>	0.011	1.163	<i>d</i>	0.007	0.237	<i>g</i>	0.004	0.937
				<i>e</i>	0.012	1.505	<i>h</i>	0.007	0.951
Mammary gland	<i>a</i>	0.442	3.018	<i>c</i>	0.741	32.765	<i>f</i>	0.344	9.368
	<i>b</i>	0.208	2.525	<i>d</i>	0.098	0.606	<i>g</i>	0.079	2.045
				<i>e</i>	0.142	32.173	<i>h</i>	0.061	2.023
" tumor				<i>c</i>	0.030	0.383			
				<i>d</i>	0.045	0.212			
				<i>e</i>	0.768	1.440			
	<i>a</i>	0.0009	0.257	<i>c</i>	<0.002	<7.021	<i>f</i>	0.003	0.402
	<i>b</i>	0.001	0.955	<i>d</i>	0.001	0.317	<i>h</i>	0.001	1.475
Preputial glands				<i>e</i>	0.001	0.720			
Various tissues									
Skeletal muscle	<i>a</i>	0.650	0.225	<i>c</i>	0.362	0.362	<i>f</i>	1.401	0.754
	<i>b</i>	1.182	0.393	<i>d</i>	0.777	0.135	<i>g</i>	0.718	0.800
				<i>e</i>	7.347	2.120	<i>h</i>	0.835	0.882
Cardiac "	<i>a</i>	0.005	0.134	<i>c</i>	0.003	0.255	<i>f</i>	0.004	0.103
	<i>b</i>	0.014	0.406	<i>d</i>	0.013	0.172	<i>g</i>	0.005	0.540
				<i>e</i>	0.029	0.762	<i>h</i>	0.011	0.871

TABLE II—*Concluded*

	Crude iodo- $\alpha$ -estradiol in normal (non-tumor-bearing) mice			Crude iodo- $\alpha$ -estradiol in mammary tumor-bearing mice			Diiodo- $\alpha$ -estradiol in normal (non-tumor-bearing) mice		
	Animal	Per cent of injected dose	Concentration*	Animal	Per cent of injected dose	Concentration*	Animal	Per cent of injected dose	Concentration*
Various tissues— <i>continued</i>									
Skin	<i>a</i>	6.222	5.531	<i>c</i>	0.851	2.340	<i>f</i>	2.854	2.483
	<i>b</i>	7.819	8.881	<i>d</i>	11.157	5.875	<i>g</i>	0.481	1.716
Fat				<i>e</i>	1.643	2.039	<i>h</i>	0.550	2.100
	<i>a</i>		0.614	<i>c</i>		2.191	<i>f</i>		1.349
	<i>b</i>		28.641	<i>d</i>		0.660	<i>g</i>		1.540
				<i>e</i>		2.004	<i>h</i>		1.201
Bone				<i>c</i>	<0.002	<0.020	<i>f</i>	0.018	0.076
	<i>b</i>	0.104	0.513	<i>d</i>	0.047	0.125	<i>g</i>	0.009	0.278
							<i>h</i>	0.007	0.132
Brain	<i>a</i>	0.004	0.042	<i>c</i>	<0.002	<0.064	<i>f</i>	0.004	0.025
	<i>b</i>	0.006	0.056	<i>d</i>	0.006	0.031	<i>g</i>	0.004	0.122
				<i>e</i>	0.016	0.016	<i>h</i>	0.011	0.885
Site of injection	<i>a</i>	0.273		<i>d</i>	0.354		<i>f</i>	0.192	
	<i>b</i>	0.161		<i>e</i>	27.437		<i>g</i>	15.674	
							<i>h</i>	34.258	
Carcass	<i>a</i>	0.588	0.237	<i>c</i>	0.279	0.319	<i>f</i>	0.980	0.421
	<i>b</i>	1.579	0.256	<i>d</i>	1.406	0.256	<i>g</i>	0.891	1.301
				<i>e</i>	2.086	0.896	<i>h</i>	0.630	0.771

All Geiger-Müller counts below 10 (10 counts being approximately 3 times the standard error of the background) were made equal to 10 and, after the proper calculations, are preceded by <.

\* The concentration is expressed as the number of counts per mg. of organ weight over the number of counts recovered in the animal's body after exclusion of the site of injection, gastrointestinal tract, feces, and urine per mg. of body weight.

of the gastrointestinal tract, and in the feces where on the average 30 per cent of the injected dose was present. The liver contained a moderate concentration, which was on the average less than that of the bile. Among the remaining glands related to the gastrointestinal tract, the submaxillary showed the highest concentration.

An average of about 9 per cent of the injected dose was excreted in the urine, while the values for the kidney were rather low.

A significantly high concentration was found in mammary glands, fat, and skin. In the mammary tumors, the concentration approximated the lower limit of the amount found in normal mammary tissue. The concentration in other organs and tissues was low, with the possible exception of vagina, uterus, and adrenals. Other endocrine glands also showed rather

low values. However, a high concentration of radioactivity was found in the thyroid which contained from 0.02 to 0.29 per cent of the injected dose.

The high concentration of radioactivity found in the skin was confirmed by the method of radioactive autography with frozen sections of the dorsal skin coated with photographic emulsion (21). A large amount of water-insoluble radioactivity was thus observed in the derma, a small amount in the hypodermis, and none in the epidermis or hair follicles (Fig. 1).

The animal bearing the advanced mammary tumor (Animal *e*) showed more radioactivity than the other animals in most organs and tissues, especially in blood, jejunoleum and contents, liver, mammary tumor, and skeletal muscle, while the amount of radioactivity excreted in the feces was much lower than with the other animals.



FIG. 1. Autograph of the skin of Animal *c*. The skin from the back was fixed in neutral formalin, sectioned on the freezing microtome, coated with photographic emulsion, and exposed in the dark. The black lines in the epidermis (top of photograph) are artifacts. The smooth darkening of the derma indicates a high concentration of radioactivity, while the light graying in the hypodermis (lower part of photograph) points to a low concentration of radioactivity.  $\times 58$ .

In most animals, very little remained at the site of injection following the termination of the experiments. However, the animal bearing the advanced mammary tumor (Animal *e*) and the animals receiving the iodinated  $\alpha$ -estradiol in the ethanol-saline-Duponol vehicle (Animals *g* and *h*) still retained 27, 15, and 34 per cent of the injected dose respectively in this region.

A second experimental series was devised to determine whether there were sex differences in the distribution of crude radioiodo- $\alpha$ -estradiol. 1.7  $\gamma$  of the iodinated  $\alpha$ -estradiol were administered subcutaneously into the left thigh of each of three male C3H mice weighing about 28 gm. and each of three C3H female mice weighing about 24 gm. The experimental procedure in this and subsequent series was the same as in the preceding experiment. The content of radioactivity in liver, intestine, feces, and urine was

found to be similar in both sexes (Table III). The amount of radioactive material found in the jejunioileum and contents and in the colon-cecum and contents was somewhat lower in females than in males, but the difference is of doubtful significance.

TABLE III

*Influence of Sex on Radioiodo- $\alpha$ -estradiol Content of Liver, Intestine, Feces, and Urine*

Organ	Males		Females	
	Per cent of injected dose	Concentration*	Per cent of injected dose	Concentration*
Jejunum, ileum, and contents	4.810	0.683	0.627	0.067
	4.080	0.643	2.460	0.296
	1.424	0.237	2.896	0.255
Average.....	3.438	0.521	1.994	0.206
Colon, cecum, and contents	5.835	1.369	1.751	0.350
	7.143	1.755	4.426	0.868
	2.892	0.653	2.552	0.499
Average.....	5.290	1.259	2.910	0.572
Feces	37.291	10.578	39.358	12.198
	40.695	14.195	36.514	19.155
	47.391	16.224	46.737	11.547
Average.....	41.792	13.666	40.870	14.300
Liver	0.821	0.140	0.428	0.070
	0.946	0.161	0.867	0.135
	0.586	0.092	0.642	0.083
Average.....	0.785	0.131	0.646	0.096
Urine	10.656		3.477	
	4.912		15.963	
	3.693		2.913	
Average.....	6.420		7.451	

\* The concentration is expressed as the number of counts per mg. of organ weight over the injected number of counts per mg. of body weight.

In the males, the testes showed an average concentration of radioactivity of 0.030 and the seminal vesicles, 0.139; the latter value varied as the weight of the glands and presumably was dependent on the amount of secretion present. The prostate showed an average concentration of 0.152, but since



TABLE IV

*Influence of Pregnancy and Lactation on Radioiodo- $\alpha$ -estradiol Uptake of Mammary Gland, Liver, and Fat*

Organ	Normal controls		Pregnant		Lactating	
	Per cent of injected dose	Concentration*	Per cent of injected dose	Concentration*	Per cent of injected dose	Concentration*
Liver	1.029	0.102	1.309	0.234	1.145	0.230
	0.991	0.184	1.367	0.212	1.629	0.204
	0.687	0.134	1.369	0.158	1.146	0.123
	0.704	0.100	1.988	0.255	1.885	0.135
	1.221	0.155	1.855	0.242	0.615	0.084
	0.773	0.120	1.755	0.234	0.889	0.113
Average.....	0.901	0.133	1.607	0.223	1.135	0.148
Mammary gland	0.106	0.210	0.258	0.210	0.032	0.032
	0.106	0.083	0.373	0.190	0.380	0.162
	0.087	0.078	0.440	0.205	0.535	0.132
	0.074	0.059	0.474	0.301	1.130	0.303
	0.126	0.166	0.304	0.279	0.052	0.046
	0.089	0.093	2.409	1.682	0.069	0.036
Average.....	0.098	0.115	0.710	0.478	0.366	0.119
Fat 1, interscapular		0.049		0.087		0.054
		0.044		0.064		0.088
		0.036		0.076		0.084
		0.033		0.117		0.055
		0.042		0.039		<0.023
		0.050		0.303		<0.055
Average.....		0.042		0.114		<0.060
Fat 2, periuterine		0.211		0.216		<0.036
		0.035		0.130		0.044
		0.046		0.049		<0.093
		0.016		0.099		<0.060
		0.044		0.097		<0.054
		0.043		0.066		<0.038
Average.....		0.066		0.110		<0.054

All Geiger-Müller counts below 10 (10 counts being approximately 3 times the standard error of the background) were made equal to 10 and after the proper calculations are preceded by <.

\* The concentration is expressed as the number of counts per mg. of organ weight over the injected number of counts per mg. of body weight.

part of the urethra was included in the prostatic mass, the samples may have been contaminated with urine.

Because of the high concentration of radioactivity found in mammary glands, it was decided to investigate the ability of these glands to collect the radioactivity under various physiological conditions. 1.8  $\gamma$  of crude radioiodo- $\alpha$ -estradiol were administered subcutaneously into the left thigh of each of eighteen hybrid female albino mice which had been divided into three groups. The first group consisted of normal controls weighing 20 to 23 gm., the second, of pregnant mice weighing 31 to 33 gm., and the third of lactating mice weighing 28 to 30 gm. All animals were sacrificed 10 to 12 hours after the injection. The mammary glands of each animal were digested together, with the exception of the inguinal gland adjacent to the injection site. The discarded gland comprised less than one-fourth of the total mammary tissue.

Pregnancy produced a definite increase in the ability of liver, fat, and especially mammary glands to collect the administered radioactive material (Table IV). The mammary glands of pregnant animals showed at least a 2-fold increase in concentration over that found in the normal controls, even after the high figure of 1.682 was eliminated. Lactation, on the other hand, tended to depress the concentration of radioactivity found in liver, mammary glands, and fat to the control level.

In the pregnant females, the uterine contents showed a moderate amount of radioactivity with an average concentration of 0.101 in the placentas and 0.090 in the fetuses.

*Fractionation of Organs*—In order to obtain information on the nature of the radioactive material present in tissues and excreta, the alkaline digests were neutralized with 50 per cent hydrochloric acid, acidified with a further 10 volumes per cent excess of concentrated hydrochloric acid, and hydrolyzed by boiling for 10 minutes (22-24). After cooling, the material was extracted three times with 10 cc. portions of freshly distilled ether. The combined ether extracts were washed with two 10 cc. portions of water, evaporated to dryness, and dissolved in 2 cc. of NaOH for counting as above. The aqueous phase was made alkaline with NaOH and an aliquot counted similarly. In a number of cases, the acidified digest was fractionated without hydrolysis (Table V).

Fractionation of the material prepared for injection revealed that on the average 93 per cent of the radioactivity was extractable with ether (Table V, Column 2). Acid hydrolysis, on the other hand, caused some cleavage of iodine which appeared in the water-soluble fraction (Table V, Column 1). An alkaline digest of any tissue with the possible exception of the stomach, when added to the sample of radioiodo- $\alpha$ -estradiol, prevented its destruction by hydrolysis (Table V, Column 1).

TABLE V  
Percentage of Radioiodo- $\alpha$ -estradiol of Organs and Excreta Present  
in Ether-Soluble Fraction

In vitro experiments with radioiodo- $\alpha$ -estradiol	Hydro- lyzed (1)	Non- hydro- lyzed (2)	Organs from mice treated with radioiodo- $\alpha$ -estradiol	Hydro- lyzed (3)	Non- hydro- lyzed (4)
Sample of radioiodo- $\alpha$ - estradiol as prepared for injection	77	92	Stomach and contents	21	19
	53	88		11	14
	71	97		48	44
	80	95		39	32
Same + stomach and contents	90	96	Duodenum and con- tents	47	41
	76	87		85	82
	65	91		67	64
Same + duodenum and contents	82	84		54	56
				75	57
				83	70
				81	74
Same + jejunioileum and contents	94	95	Jejunioileum and con- tents	78	70
				76	70
				71	70
				64	61
				61	57
Same + colon-cecum and contents	91	92	Colon-cecum and con- tents	71	57
				75	76
				62	65
				70	73
				46	43
Same + feces			Feces	54	58
	87	82		67	69
	82	84		50	50
	81	81		64	65
				57	60
				38	38
" + liver			Liver	32	34
	87	94		61	51
	97	89		64	47
	83	86		68	63
				64	62
				53	55
" + mammary glands			Mammary glands	56	52
	82	86		64	54
				62	52
Same + fat	86	87	Fat	43	39
			Urine	55	54
				39	37
				44	31
				24	20
				31	24
				61	47
				53	38

Fractionation of the organs from the experimental animals (Table V, Columns 3 and 4) revealed that about two-thirds of the radioactivity in the stomach was present in an ether-insoluble form. The other organs contained at least half of their activity in an ether-soluble form. Hydrolysis slightly increased the activity in the ether fraction in the case of the duodenum and contents, jejunum and contents, liver, urine, and mammary glands, while the activity in the colon-cesum and contents and in feces appeared to be unaffected by hydrolysis. The changes, however, were not statistically significant. Ether extraction without hydrolysis revealed that 58 per cent of the radioactivity in the seminal vesicles and 18 per cent of the radioactivity of a fetus were in an ether-soluble form.

*Metabolism of Iodo- $\alpha$ -estradiol*—The rôle of liver and gastrointestinal tract in the metabolism of the iodinated  $\alpha$ -estradiol was examined after ligation of the common bile duct in four A strain female mice weighing 24 to 30 gm., while a sham operation was performed in four similar controls. Each animal received a subcutaneous injection of 3.5  $\gamma$  of radioiodo- $\alpha$ -estradiol.

Following the ligation of the common bile duct (Table VI), there was an increase in the radioactivity of the liver, plasma, and urine, a marked diminution of the activity in the jejunum and contents, and in the colon-cesum and contents, and no activity in feces. Furthermore, fractionation of the urine after hydrolysis revealed an increase of 30 per cent in the radioactivity of the ether fraction after bile exclusion.

In order to determine whether the radioiodo- $\alpha$ -estradiol would be absorbed from the gastrointestinal tract, 7  $\gamma$  were injected directly into the stomach of four A strain female mice weighing 20 to 25 gm. The results (Table VII) showed that absorption of the material from the gastrointestinal tract could take place, since blood plasma, thyroid, liver, uterus, submaxillary gland, mammary glands, and urine showed an amount of activity comparable to that found after subcutaneous injection (Table II). Some of the material was still bound in organic combination, since the ether fraction of the liver contained 19 per cent of the radioactivity present in this organ. These values were, however, lower than in normal animals (Table V).

A preliminary attempt was made to determine, with the help of biological assays, whether the intestinal excretion of pure  $\alpha$ -estradiol is similar to that of its iodinated prototype. Ten female A mice received 100  $\gamma$  of  $\alpha$ -estradiol each by subcutaneous injection and were sacrificed 10 hours later. Three bioassays were carried out, first with jejunum and contents, second with the colon-cesum and contents, and third with the feces of the ten animals. The material was digested in 2 N NaOH, acidified, extracted five times with ether washed with a 5 per cent solution of NaHCO<sub>3</sub> and then with

water, taken to dryness, and finally dissolved in corn oil. Five spayed female rats were used for each sample tested. Each animal was given 6 per cent of the total extract. Negative results were obtained with the jejuno-

TABLE VI

*Distribution of Iodo- $\alpha$ -estradiol after Common Bile Duct Ligation*

The figures show the radioiodine content of organs and excreta expressed as percentage of the injected dose.

	Blood plasma	Stomach and contents	Duodenum and contents	Jejunum, ileum, and contents	Colon, cecum, and contents	Feces	Liver	Urine
Controls, sham operation	0.26	2.11	1.09	7.45	8.21	18.67	0.89	1.93
	0.21	2.60	0.95	5.23	5.68	26.19	0.82	11.44
	1.38	3.45	1.09	7.16	11.39	34.60	0.99	3.24
	0.54	2.68	0.97	12.62	16.73	14.95	1.38	15.51
Average.....	0.60	2.71	1.03	8.12	10.50	23.60	1.02	8.03
Bile duct ligated	0.94	5.32	0.45	2.44	0.47	0.02	4.91	30.74
	0.94	1.25	0.13	1.95	0.34	0.00	1.30	12.92
	1.52	1.42	0.79	2.38	0.60	0.01	2.87	25.62
	1.05	0.22	4.51	1.92	0.75	0.00	1.29	36.01
Average.....	1.11	2.05	1.47	2.17	0.54	0.01	2.59	26.32

TABLE VII

*Distribution of Iodo- $\alpha$ -estradiol after Its Intragastric Instillation*

The figures show the radioiodine content of organs and excreta as percentage of the injected dose.

	Blood plasma	Stomach and contents	Duodenum and contents	Jejunum, ileum, and contents	Colon, cecum, and contents	Feces	Liver	Submaxillary glands	Urine	Thyroid	Uterus	Mammary glands
	0.28	1.19	0.51	1.34	2.71	7.62	0.39	0.10	8.20	1.30	0.01	0.06
	0.89	30.97	0.58	9.88	11.51		0.47	0.10	12.33	1.79	0.04	0.08
	0.15	1.93	0.13	1.98	4.94	16.24	0.35	0.05	1.26	2.23	0.01	0.09
	0.44	3.92	0.14	4.48	4.35	14.95	0.52	0.28	24.53	1.53	0.03	0.09
Average.....	0.44	9.50	0.34	4.42	5.88	12.94	0.43	0.13	11.58	1.71	0.02	0.08

ileum and colon-cecum, while the feces gave a 100 per cent response. However, another group of five rats given 1.2 per cent of the feces samples showed uniformly negative results. The amount excreted expressed as

$\alpha$ -estradiol was calculated to be between 2.5 and 12.5  $\gamma$ ; that is to say, between 0.25 and 1.25 per cent of the injected dose.

#### DISCUSSION

The results of the isotope dilution (Table I) indicated that the crude radioactive injection material consisted of monoiodo- $\alpha$ -estradiol and diiodo- $\alpha$ -estradiol, with some residual  $\alpha$ -estradiol. This material was found to be partitioned between ether, 10 per cent sodium carbonate solution, and 1 N sodium hydroxide in the same way as  $\alpha$ -estradiol. Therefore, iodination of  $\alpha$ -estradiol resulted in the fixation of 1 or 2 atoms of iodine in the molecule without affecting its phenolic nature. The identification of 2,4-dibromo- $\alpha$ -estradiol after the action of N-bromoacetamide on estradiol (19) makes it reasonably certain that the diiodo derivative obtained by the action of either N-iodoacetamide or  $I_2$  on  $\alpha$ -estradiol also had the halogen in the 2,4 positions.

As far as can be judged from the scanty information available in the literature, the metabolism of iodinated  $\alpha$ -estradiol was, with the exception of the large fecal excretion, similar to that of natural estrogens, but different from that of artificial ones. Thus the resorption of the iodo- $\alpha$ -estradiols from the site of injection must have been quite rapid, since little or no activity remained there at 10 to 12 hours after the injection, except in the case of Animal *e* whose metabolism had apparently been slowed by an advanced mammary tumor and in the two animals (Animals *g* and *h*) in whose case a detergent was added to the injection material (Table II). Reports from the literature indicated that the natural estrogens, estrone and  $\alpha$ -estradiol, but not their esters or artificial estrogens, were rapidly resorbed after subcutaneous injection (15, 16). Similarly, the small amount of radioactivity found in the blood suggested a quick turnover of the iodinated  $\alpha$ -estradiol, comparable to that noted with natural estrogens (5-8, 24) but unlike that of the artificial estrogens (16, 17).

The withdrawal of the material from the blood was effected by three different routes; namely, the liver, the gastrointestinal tract, and the kidney. The considerable amount of radioactivity in the lumen of the digestive tube and in the feces arose from the secretion by way of the bile and the gastrointestinal tract itself. The bile was the main source, contributing 90 per cent of the radioactivity found in the digestive tract. This was demonstrated in the bile duct-ligated animals (Table VI) in which only 6.24 per cent of the radioactivity was found in gastrointestinal tract and feces, as compared with 45.96 per cent in the normal animals. A recent report on equilin brominated with radiobromine showed that it behaved like the iodinated  $\alpha$ -estradiol, since 35 per cent of the injected dose was found in the intestinal contents 6 hours after injection (25). Similarly, in the case of

natural estrogens, a large proportion of an injected dose is excreted in the bile (12-14). Preliminary data on ether extraction of the bile showed no increase of the ether-soluble radioactivity after acid hydrolysis, thus suggesting that the labeled material in the bile was not conjugated, in confirmation of previous work with natural estrogens (26). On the other hand, the liver, through which large amounts of the labeled compound must have passed before being excreted into the bile, showed only a moderate concentration (Table II), as is the case with natural estrogens (5, 6), while in contrast the liver content was found to be very high after administration of artificial estrogens (11, 17).

The large amounts of labeled material found in gastrointestinal content and feces did not give a true figure of the biliary and gastrointestinal secretion, since appreciable resorption from the digestive tube must take place. This was shown by the entry of radioactivity into the organs after the intragastric administration of radioiodo- $\alpha$ -estradiol (Table VII). It may be concluded that there is an interplay of elimination into and resorption from the intestinal lumen.

The excretion of estrogenic material in the feces has been observed under physiological conditions (27-29), especially in pregnancy (28, 30, 31), and also after the administration of estrogens (11, 27, 28, 32). However, the amount recovered was rather small. In our experiments with pure  $\alpha$ -estradiol, between 0.25 and 1.25 per cent of the injected dose was recovered, by bioassay of the feces, 10 hours after injection. In contrast, about 30 per cent of the injected iodo- $\alpha$ -estradiol was present in feces, of which more than half was in an ether-soluble form. The difference between the fecal recoveries may indicate a difference in behavior of the iodo- $\alpha$ -estradiol and natural estrogens. More likely, however, estrogen catabolites were also excreted in large amounts in the feces, but in a biologically inactive form. In this regard, 30 per cent of the radioactivity in the feces was found to be partitioned with the acids and 25 per cent with the phenols.

The third path for the elimination of radioactivity from the blood was via the urinary system, since 3.5 to 24.8 per cent of the injected radioactivity was found in the urine, a third of which was in ether-soluble form. Similar values, following the administration of natural estrogens, had been previously reported by many investigators (3-7, 11, 12, 16, 23, 27-29, 32).

Despite the wide-spread distribution of radioactivity following the administration of radioiodo- $\alpha$ -estradiol (Table II), it was found that, aside from bile, gastrointestinal tract, and thyroid, only very few organs actually fixed radioactivity in excess of what would be expected from a purely random distribution (the concentration was calculated in such way that a value of 1 represented such a distribution). Thus, organs having a concentration of radioiodine above 1, namely mammary glands, skin, sub-

maxillary glands, liver, and fat, were assumed to have concentrated the injected material, while those showing a figure below 1 were taken as showing no preferential distribution (Table II).

On the whole, the mammary glands showed a higher concentration of radioactivity than any other parenchymatous organ. This uptake was mostly due to mammary tissue proper and not surrounding fat, since the latter showed a lower concentration of radioactivity (Table IV).

On the other hand, other estrogen end-organs, namely uterus, cervix, and vagina, showed an unexpectedly low concentration of radioactivity. However, the concentration of radioactivity found in the vagina was in keeping with the minute doses of estrogen required to produce a local effect (33, 34).

Mammary cancers showed a lower concentration of radioactivity than the healthy mammary gland. It was further noted that the non-cancerous mammary tissue of the cancer-bearing animals was more active than the normal in fixing the radioactive material in two out of three animals bearing tumors.

In most organs over one-half of the radioactivity was present in an ether-soluble form. Furthermore, a 10 minute acid hydrolysis (35, 22, 23) slightly increased the yield of ether-soluble compounds in mammary glands, liver, duodenum and contents, jejunoleum and contents, and urine (Table V), possibly indicating that a small amount of conjugated material was present.

Since only a small fraction, if any, of the radioactivity in the aqueous fraction was conjugated, the remaining radioiodine was in the form of iodide or simple organic iodo compound released from the labeled steroid. The former possibility is supported by the presence of some radioactivity in the thyroid (Table II), since so far no iodine compound other than iodide has been shown to enter this gland (36). The presence of iodide in this gland, therefore, implies a breakdown of the labeled steroid. One of the possible sites of this breakdown is the gastrointestinal tract, since there was a greater increase in iodide concentration in the thyroid when the labeled estradiol was given by the intragastric route (Table VII).

If one bears in mind that the method for separation gives results that are about 19 per cent too low, it may be calculated from the over-all average of the available figures (Table V) that 32 per cent of the iodo- $\alpha$ -estradiol has been broken down to a water-soluble iodine compound within 10 hours after administration.

#### SUMMARY

1.  $\alpha$ -Estradiol was iodinated with radioiodine under conditions such that about half the iodine was substituted as mono- and half as diiodo- $\alpha$ -estradiol (Table I). By the vaginal smear test these iodinated compounds proved to be physiologically inert at the dose level of 100  $\gamma$ . Such iodo- $\alpha$ -estradiol



was administered subcutaneously to mice in doses varying from 1.7 to 18.0  $\gamma$ . Some animals were treated with pure diiodo- $\alpha$ -estradiol in doses of 0.5 or 2.0 mg. After 10 to 12 hours the distribution and excretion of the radioactivity were examined in about forty organs and tissues (Table II). Ether extraction of organs and excreta was carried out to determine the state of the recovered radioactivity (Table V).

2. The outstanding feature of the metabolism of the iodinated  $\alpha$ -estradiol in both male and female mice is the accumulation of labeled material in the gastrointestinal tract (Tables II and III), entry into which is predominantly via the liver and bile (Table V). A large fraction of the material is excreted in the feces. Some, however, must have been resorbed from the intestinal lumen, since intragastric instillation of the iodinated steroids leads to accumulation of radioactivity in many organs and in the urine (Table VI).

3. The highest concentration of radioactivity is usually found in the mammary glands. In pregnancy, the accumulation is markedly increased, while a return to normal levels occurs during lactation (Table IV). The uptake in mammary tumors is less than that of normal mammary tissue.

4. The other accessory sex organs, uterus, cervix, and vagina, fix only small amounts of radioactivity. Much greater deposition is observed in the skin, especially in the derma (Fig. 1).

5. Acid hydrolysis indicates that only a small part, if any, of the radioactivity is combined in a conjugated form in the small intestine, liver, mammary glands, and urine, but not in feces (Table V).

6. A survey of the literature reveals that unesterified estrone and  $\alpha$ -estradiol (but not the artificial estrogens) show rapid resorption from the site of injection, swift departure from the blood plasma, entry into red blood cells, marked excretion in the bile, and moderate excretion in the urine, mainly in a conjugated form. The same results were obtained with iodinated  $\alpha$ -estradiol in the present work. However, the large fecal excretion found with the labeled material has not been observed with natural estrogens by bioassay, probably because their fecal metabolites are biologically inactive.

This work was supported by grants from the Donner Foundation, the National Research Councils (Ottawa and Washington), and the Blanche Hutchison Fund of McGill University. The technical help of Miss J. Cambron is acknowledged.

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SYNTHESIS OF A POLYSACCHARIDE OF THE STARCH-  
GLYCOGEN CLASS FROM SUCROSE BY A CELL-  
FREE, BACTERIAL ENZYME SYSTEM  
(AMYLOSUCRASE)\*

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(Received for publication, July 28, 1948)

The present paper deals with the conversion of sucrose to an amylopectin- or glycogen-like polysaccharide by means of a cell-free bacterial enzyme system obtained from *Neisseria perflava*, a Gram-negative coccus that occurs in the throats of healthy people. This reaction is of interest, not only because of its possible bearing on the well known interrelationship between sucrose and starch in the plant world, but also because of its apparent freedom from mediation by glucose-1-phosphate, which is commonly believed to be the essential substrate for the synthesis of all members of the starch-glycogen class.

We have already reported (1, 2) that cultures and resting cell suspensions of *Neisseria perflava* convert sucrose into a polysaccharide with the chemical properties of amylopectin; large amounts are formed from sucrose, smaller amounts from glucose-1-phosphate, but none at all from other common sugars. The enzyme system responsible for the conversion of sucrose to polysaccharide is, in addition, obtainable in cell-free form (2). The present paper gives a method for obtaining the enzyme solutions from the bacterial cultures, and deals with the action of these enzyme solutions on sucrose and on glucose-1-phosphate and with the chemical properties of the products of their action on sucrose.

EXPERIMENTAL

*Preparation of Enzyme Solutions*

Strain 19-34 of *Neisseria perflava*, known to produce large amounts of amylopectin-like material from sucrose, was cultivated in broth comprising 0.1 per cent Bacto-peptone, 0.15 per cent sodium citrate, 0.02 per cent yeast extract, 0.06 per cent  $\text{KH}_2\text{PO}_4$ , 0.15 per cent  $\text{Na}_2\text{HPO}_4$ , and 0.05 per cent glucose. Flasks containing 800 ml. of the medium were inoculated with 10 ml. of a 1 day culture grown in the same medium, and incubated

\* Aided by a grant from the Sugar Research Foundation, Inc.

at 37° for 5 days. In order to precipitate the enzymes, each flask of culture was treated with 300 gm. of ammonium sulfate and the mixture centrifuged in the cold for 1 hour at 1500 R.P.M. The sediments, which contained the bacterial cells as well as the enzymes, were separated from the supernatant fluids, drained, and further freed from phosphates and other soluble constituents of the culture medium by washing in half saturated ammonium sulfate solution that had been adjusted to pH 6.4 with ammonia. The washed precipitates were extracted with 0.025 M maleate buffer (pH 6.4) by using 15 ml. of buffer for each 800 ml. of original culture, and the extracts were clarified in a 14 inch angle-head centrifuge at 2500 R.P.M. for 2 hours. The final solutions were entirely free of bacterial cells and cell fragments; and, from the many control tests made on the incubated enzyme-substrate mixtures, we are certain that all the reactions observed in the present paper occurred in the complete absence of bacteria.

*Action of Neisseria Enzymes on Sucrose and on Glucose-1-phosphate*

*Material and Methods*—Mixtures of enzyme plus sucrose or glucose-1-phosphate buffered at pH 6.4 were used throughout. The sucrose was a selected sample of beet sugar essentially free of the traces of dextran, amylopectin, and other alcohol-precipitable material present in many lots of reagent and commercial sucrose (3, 4). The glucose-1-phosphate was the dipotassium salt prepared from an enzymatic digest of potato starch by the method of McCready and Hassid (5); though recrystallized, it contained a trace of accompanying starch.

The enzyme-substrate mixtures were examined for opalescence, turbidity on addition of 2.0 volumes of alcohol, kind and intensity of color with iodine, and contents of free reducing sugar, polysaccharide, inorganic phosphorus, and total acid-soluble phosphorus. For determining the intensity of color with iodine, 0.1 ml. of 1 per cent iodine and 2 per cent potassium iodide was added to 5.0 ml. of a 1:10 dilution of the enzyme-substrate mixture and measurements were made with a Klett-Summerson photoelectric colorimeter with green Filter 54 (spectral range 500 to 570  $m\mu$ ); the zero point was the iodine-iodide reagent blank. Free reducing sugar contents of the mixtures were determined by the Hagedorn and Jensen (6) method and are expressed as mm of fructose<sup>1</sup> per liter. For the polysaccharide determinations, 2.0 ml. of the enzyme-substrate mixture were placed in a large test-tube and treated with 8.0 ml. of 0.6 M acetate buffer (pH 5.0) and 20.0 ml. of alcohol. (Adjustment to pH 5.0 was essential in order to prevent precipitation of glucose-1-phosphate from systems containing that sugar.) After storage overnight at 4°, the mixture was centrifuged; 2.0 ml. of 1.0 N HCl were added to the precipitate, and the

<sup>1</sup> Evidence that the free reducing sugar is fructose and that the alcohol-precipitable material is a polyglucoside will be presented later in the paper.

tube fitted with an air condenser and immersed in boiling water for 2.5 hours. Reducing sugars were determined on the neutralized hydrolysate by the Hagedorn and Jensen (6) method and calculated as mm of glucose<sup>1</sup> per liter. The contents of inorganic phosphorus and of total acid-soluble phosphorus after mineralization with sulfuric and nitric acids were determined by the Fiske and Subbarow (7) procedure. All figures given for the production of polysaccharide, free reducing sugar, or inorganic phosphorus represent the difference between the contents determined before and after incubation of the enzyme-substrate mixtures.

*Over-All Action upon Sucrose*—A representative experiment showing the action of the *Neisseria* enzymes upon sucrose is shown in Table I. Equal

TABLE I  
*Changes Produced by Action of Neisseria Enzymes upon Sucrose*

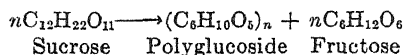
Time of incubation at 10°	Opalescence	Ppt. with alcohol	Intensity of color with iodine	Polysaccharide produced*	Fructose liberated*
hrs.				mM	mM
0	±	±	6	0.0†	0.0†
3	+	+	27	1.1	1.0
6	++	++	58	2.0	2.2
12	+++	+++	100	4.2	4.2
24	++++	++++	173	7.8	7.8
48	+++++	+++++	250	13.8	14.0
96	+++++	+++++	370	24.1	25.6

\* Since the initial concentration of the sucrose substrate was 100 mM, the values correspond to the per cent conversion of sucrose to polysaccharide and fructose.

† The unincubated mixture contained 0.3 mM of reducing sugar and 0.6 mM of polysaccharide, calculated as hexose; the nature of the reducing substance and polysaccharide is unknown.

volumes (250 ml.) of enzyme solution and of 0.2 M sucrose in 0.025 M maleate buffer, pH 6.4, were mixed and maintained at 10°. At regular intervals, small samples were removed and examined for opalescence, precipitation with alcohol, color with iodine, and contents of polysaccharide and free reducing sugar.

Beginning with the first observations at 3 hours, there was an orderly increase in opalescence, in amount of alcohol-precipitable material, and in amounts of polysaccharide and of free reducing sugar. At each period there was excellent agreement between the quantity of polysaccharide and of free reducing sugar that had been produced, indicating that the over-all equation for the action of the *Neisseria* enzymes upon sucrose, as previously suggested (1, 2), is:



The enzyme-sucrose mixture also showed an increasingly intense maroon coloration on treatment with iodine. However, it was noted that after the 1st day the increase in intensity of the color with iodine did not keep pace with the increases in polysaccharide and reducing sugar contents. This phenomenon, which probably is the result of alteration of the newly formed polysaccharide by some enzyme present in the *Neisseria* extracts, is being further investigated.<sup>2</sup>

*Influence of High Concentrations of Phosphate*—Although entirely non-reactive with glucose, glucose plus fructose, or other common sugars, the enzyme preparations, as previously found for the living bacteria, synthesized polysaccharide from glucose-1-phosphate. In the case of whole bacterial cells, the synthesis from glucose-1-phosphate was suppressed by addition of high concentrations of inorganic phosphate which did not inhibit the synthesis from sucrose (1, 2). A similar experiment was made with the cell-free enzyme system.

Four enzyme-substrate mixtures were prepared: 5.0 ml. of enzyme solution were added to 5.0 ml. of (1) 40 mM sucrose in 320 mM maleate buffer, (2) 40 mM glucose-1-phosphate in 320 mM maleate buffer, (3) 40 mM sucrose in 320 mM phosphate buffer, and (4) 40 mM glucose-1-phosphate in 320 mM phosphate buffer. The mixtures were held at 10° for 24 hours and then analyzed. The results are given in Table II.

In the systems buffered with maleate, polysaccharide was formed from glucose-1-phosphate as well as from sucrose, but in the case of the synthesis from sucrose the low content of total P and the absence of any change in the inorganic phosphate make it unlikely that glucose-1-phosphate served as an intermediate substance in that reaction. More conclusive evidence is presented by the data from the systems buffered with phosphate. In these systems, which contained 8 moles of phosphate to 1 of substrate, the synthesis from glucose-1-phosphate was entirely suppressed, whereas the synthesis from sucrose was not inhibited at all. That the inhibition observed in the glucose-1-phosphate system was not due to any loss of the substrate or to any inactivation of the enzyme was proved by supple-

<sup>2</sup> It is unlikely that the alteration was due to hydrolysis by an  $\alpha$ - or  $\beta$ -amylase, since the reducing sugar liberated in the enzyme-sucrose mixture did not exceed the amount expected from a sucrose condensation reaction; nor did it contain any appreciable amount of aldose, *e.g.* maltose. It also is unlikely that phosphorolysis was involved, since the mixture did not contain any appreciable amount of free inorganic phosphate. The change in character of the polysaccharide product may have been due to some enzyme like the cross-linking enzymes in liver (8) and in potatoes (9) reported to bring about the synthesis of glycogen and of amylopectin, or, perhaps, to adsorption of some fatty acid. The former explanation seems more likely, since *Neisseria* extracts convert crystalline corn amylose into a glycogen-like polysaccharide without the release of reducing sugars, but fail to do so if heated.

mentary tests: at the end of the experiment, when solid sucrose was added to one portion, polysaccharide synthesis occurred at a rate equal to that in the maleate-buffered system, and chemical analyses on another portion showed that glucose-1-phosphate was present in its original concentration.

*Influence of Heat and Gas Treatment*—The data from the following experiment show that the enzyme solutions prepared from *Neisseria* contain two systems: an *amylosucrase*, active preferentially if not solely upon sucrose, and a *phosphorylase*, active upon glucose-1-phosphate but not upon sucrose.

A sample of enzyme solution was divided into three parts. The first was untreated, the second was heated at 45° for 10 minutes (pH 6.4), while the third, after adjustment to pH 5.9 with 1.0 N HCl, was exposed in a thin film to pure CO<sub>2</sub> gas for 5 minutes at 15° and atmospheric pressure, and

TABLE II

*Action of Neisseria Enzymes on Sucrose and on Glucose-1-phosphate in Absence and in Presence of High Concentrations of Inorganic Phosphate*

Sugar substrate, 20 mm	Buffer pH 6.4, 160 mm	Maroon with iodine	Poly- saccharide formed	Fructose liberated	Inorganic phosphorus liberated
			mm	mm	mm
Sucrose.....	Maleate	++++	7.5	8.3	0.00*
Glucose-1-phosphate....	"	+	3.1	0.0	3.5
Sucrose.....	Phosphate	+++	7.7	11.5	
Glucose-1-phosphate....	"	0	0.0	0.0	†

\* This mixture contained 0.10 mm of inorganic phosphorus and 0.20 mm of total acid-soluble phosphorus both before and after incubation.

† The glucose-1-phosphate content of this mixture, measured both before and after incubation by the method of Hassid and McCready (5), showed no change.

then readjusted to pH 6.4 with 1.0 N NaOH. The three enzyme solutions were tested for activity in mixtures containing final concentrations of 20 mm of sucrose and of glucose-1-phosphate in maleate buffer.

It is apparent (Table III) that heat treatment sufficient to cause almost complete loss of the capacity of the original enzyme solution to convert sucrose to polysaccharide and fructose resulted in only slight impairment of the capacity to convert glucose-1-phosphate to polysaccharide and free phosphate, whereas gas treatment<sup>3</sup> sufficient to cause over 90 per cent loss of activity for glucose-1-phosphate brought about loss of only about half of the activity toward sucrose.

<sup>3</sup> The inactivation produced by CO<sub>2</sub> in this experiment has been duplicated in other experiments with air, O<sub>2</sub>, or H<sub>2</sub> gas. The pH is apparently an important factor, since the inactivation which occurs in enzyme solutions adjusted to pH 5.9 did not occur in solutions at pH 6.4.

*Analysis of Products Formed from Sucrose by Neisseria Enzymes*—The starting material consisted of 440 ml. of the enzyme-sucrose mixture which had been incubated at 10° for 4 days in the experiment in Table I. The mixture was first partially deproteinized by shaking with chloroform and removing the chloroform emulsion layer. The fluid then was treated with 22 gm. of crystalline sodium acetate and 1.5 volumes of alcohol and was centrifuged after storage overnight at 4°. The precipitate was used for experiments on the nature of the polysaccharide, while the alcoholic supernatant fluid was used for experiments on the nature of the free reducing sugar.

*Identification of Free Reducing Sugar*—The alcoholic supernatant fluid was evaporated *in vacuo* at a temperature below 40°, and the dry residue dissolved in water. Analyses for reducing capacity (7) before and after oxidation with iodine (10) showed that all of the reducing sugar was ketose

TABLE III

*Effect of Heat and of Gas Treatment upon Activity of Enzyme Solutions for Sucrose and for Glucose-1-phosphate*

Treatment of enzyme solution	Sucrose		Glucose-1-phosphate	
	Polysaccharide	Fructose	Polysaccharide	Inorganic phosphorus
	<i>mM</i>	<i>mM</i>	<i>mM</i>	<i>mM</i>
Untreated.....	5.5	5.8	6.1	7.0
Heated at 45°, 10 min.....	0.1	0.1	5.1	6.1
Gas-treated.....	2.3	2.6	0.6	0.6

sugar; aldose was not detected. Proof that the ketose sugar was fructose was obtained by treatment of another portion of the solution with  $\alpha$ -methylphenylhydrazine sulfate, according to the method of Neuberg and Mandl (11) (the  $\alpha$ -methylphenylhydrazine sulfate was kindly supplied by Dr. Neuberg). Well formed, needle-shaped crystals of fructose methylphenylosazone, identical in appearance with those prepared from a sample of fructose, were isolated. The yield of osazone (113 mg. after recrystallization from ethyl acetate), obtained by treatment of a solution containing 92 mg. of reducing sugar, was approximately that expected from a similar amount of pure fructose (*cf.* (11)).

*Isolation and Analysis of Polysaccharide*—The alcohol-precipitated material from the enzyme-substrate mixture was extracted with 5 per cent sodium acetate solution, insoluble material was removed by centrifugation, and the solutions treated with 1.5 volumes of alcohol to precipitate the polysaccharide. Solution of the polysaccharide and precipitation with alcohol were repeated twice; the last precipitation was from a solution in distilled water at room temperature in order to insure a minimal ash



content. The final precipitate was ground under absolute alcohol and dried *in vacuo* at 25° over  $\text{CaCl}_2$ . The yield was 1.58 gm. (moisture-free basis), which represented 92 per cent of the polysaccharide present in the starting material.

The isolated product was a white amorphous powder, with an ash content (as  $\text{Na}_2\text{SO}_4$ ) of 1.2 per cent, micro-Kjeldahl nitrogen (12) 0.45 per cent, phosphorus (7) 0.03 per cent, and a reducing power (13) of less than 0.2 per cent that of glucose. Calculated on an ash- and moisture-free basis, the optical rotation was  $[\alpha]_{589}^{22} = +175^\circ$  ( $c = 0.3$  in 0.5 N NaOH); after boiling for 2.5 hours in 1.0 N HCl, with correction for the entry of water, the hydrolysate had  $[\alpha]_{589}^{22} = +49.3^\circ$  ( $c = 0.5$ ) and 91 per cent reducing sugar, as glucose. Abundant glucose phenylosazone (m.p. 205°, uncorrected) was obtained from the hydrolysate; fructose, if present at all, amounted to less than 1 per cent of the sugar, according to determinations made by the Roe procedure (14); Bial's test for pentoses was negative.

The presence of maltosidic linkages in the enzymatically synthesized polysaccharide was established by the isolation of maltose. A 382 mg. sample of ash- and moisture-free polysaccharide in 7 ml. of water was treated with 10 mg. of  $\beta$ -amylase prepared from wheat flour (15) and sufficient dilute  $\text{H}_2\text{SO}_4$  to bring the pH to 4.8; after 16 hours at 30°, the reducing value indicated that 31 per cent conversion had occurred. The digest, when treated with methanol according to the procedure described by Haworth *et al.* (16), yielded 98 mg. of crystalline  $\beta$ -maltose monohydrate,  $[\alpha]_{589}^{22} = +111.9^\circ \rightarrow +126.9^\circ$  ( $c = 1.5$  in  $\text{H}_2\text{O}$ ). The optical measurements were made with a Schmidt and Haensch polarimeter kindly made available by Dr. du Vigneaud.

The formation of the maltose through the action of  $\beta$ -amylase on polysaccharide material which had been synthesized by the action of *Neisseria* enzymes on sucrose represents the first demonstration of an enzymatic conversion of sucrose to maltose in the absence of cells. This conversion seems of fundamental interest, even though the enzymes that accomplished it were of different biological origins.

*Comparison with Other Polyglucosides*—The enzymatically synthesized polysaccharide was compared with a number of reference polyglucosides: a sample of amylopectin-like polysaccharide produced from sucrose by living cultures of the strain of *Neisseria* used in the preparation of the enzyme (1); commercial liver and oyster glycogens; purified amylopectin C-107-B and recrystallized amylose C-107/111-A prepared from a specimen of corn-starch by the pentasol method (kindly supplied by Dr. T. J. Schoch); and dextran prepared from the sucrose broth cultures of *Leuconostoc mesenteroides*, strain B (17). All of the polysaccharides were subjected simultaneously to the following procedures.

The limits of conversion to maltose by  $\alpha$ - and  $\beta$ -amylases were determined

by methods described in a succeeding paper (18); all the digestions were at 30° for 24 hours. Reactions with iodine were examined in several ways: (1) the color was observed when 5.0 ml. of a 0.04 per cent solution of polysaccharide at pH 5.8 were treated with 0.2 ml. of 1 per cent  $I_2$  and 2 per cent KI; (2) the "blue value" was determined by a slight modification (18) of the method of McCready and Hassid (19); (3) the percentage of iodine bound in complex formation (mg. of  $I_2$  per 100 mg. of polysaccharide) was measured by Schoch's modification (18) of the potentiometric method of Bates, French, and Rundle (20); and (4) the capacity of iodine to precipitate each polysaccharide from dilute (0.05 per cent) solution was tested (18). The polysaccharides were tested also for their capacities to dissolve in cold water, to "retrograde" (*i.e.*, to precipitate as particles that do not redissolve on heating) from 0.5 per cent solutions stored overnight at 8°, and to be precipitated from solution by pentasol and butanol (18). They were examined also for their opalescence in 1:1000 solution at pH 6.2, for their capacity to give serological precipitation in dilutions from 1:1000 to 1:10 million with a standardized, dextran-reactive, type 2 pneumococcus antiserum (17), and for their speed of hydrolysis by hydrochloric acid. For the latter determination, solutions of polysaccharide in 0.5 N HCl in sealed tubes were heated for 15, 20, and 45 minutes in boiling water, then cooled, neutralized, and tested for content of reducing sugar, as glucose (6). The rate of hydrolysis was expressed as the first order velocity constant,  $K_1$  (21).

From the data summarized in Table IV, the polysaccharide synthesized by the cell-free enzyme system from *Neisseria perflava* appears to be a glycogen-like substance. Although differing in that it gave much more opalescent solutions, it was similar to the glycogen from oysters and liver in respect to the extent of hydrolysis by the several  $\alpha$ - and  $\beta$ -amylases tested, the kind of color given with iodine, including the absence of any measurable "blue value," the slight amount of iodine bound in complex formation, the failure to be precipitated by iodine, pentasol, or butanol, and the likeness in the rate of hydrolysis by hydrochloric acid. In comparison to corn amylopectin it showed considerably less complete hydrolysis by the  $\alpha$ - and  $\beta$ -amylases, had less intense and less blue color with iodine, and a lower capacity to form an iodine complex. The product of the enzyme differed from the amylopectin-like polysaccharide from the living culture of the *Neisseria* in the same manner as from the corn amylopectin.<sup>4</sup>

<sup>4</sup> A further difference between the products of enzymatic and cultural source was evident from tests made by Dr. S. Hestrin of their capacity to undergo phosphorylation with purified crystalline muscle phosphorylase. He found (personal communication) that the cultural product was split to the extent of about 40 per cent, while the product formed by the cell-free enzyme underwent a reaction that was considerably smaller and slower.

TABLE IV

Comparison of Polysaccharide, Synthesized from Sucrose by Cell-Free *Neisseria Enzymes*, with Polysaccharide Elaborated by Living Cultures of Bacteria from Which Enzymes Were Derived, and with Polyglucosides from Several Other Biological Sources\*

Property	<i>N. perflava</i> polysaccharides		Source and kind of reference polyglucosides				
	Enzyme-sucrose mixture	Sucrose broth culture	Oyster glycogen	Mammalian liver glycogen	Corn amylopectin, Schoch	Corn amylose, Schoch	<i>L. mesenteroides</i> dextran
Conversion to maltose by amylases, per cent							
$\alpha$ -Amylase (saliva)	72	84	73	73	92	101	0
$\beta$ -Amylase (ungerminated barley)	40	59	37	42	57	92	0
$\beta$ -Amylase (wheat)	35	57	32	34	57	91	0
Reactions with iodine							
Color with iodine	Red-brown	Maroon	Light red-brown	Light red-brown	Maroon	Intense blue	None
"Blue value"	0	11	0	0	32	315	0
I <sub>2</sub> bound in complex formation, %	0.1 Ca.	0.2 Ca.	0.1 Ca.	0.1 Ca.	0.6	18.9	0.0
Pptn. by iodine	0	0	0	0	0	+++	0
Other properties							
Solubility in cold water	+++	+++	+++	+++	+++	±	+++
"Retrogradation" tendency	0†	0†	0	0	0	+++	0
Pptn. by pentasol or butanol	0	0	0	0	0	+++	0
Opalescence of 1:1000 solution	+++	+++	0	0	±	0	+
Serological precipitation†	0	0	0	0	0	0	+++
Acid hydrolysis ( $K_1$ ), min. <sup>-1</sup>	0.04	0.05	0.04	0.05	0.05	0.06	0.01

\* All the data are calculated to the moisture-free basis.

† Both the *N. perflava* polysaccharides do tend to precipitate out of solutions stored for long periods in the cold, but they redissolve readily without heating.

‡ 0, no precipitation in tests of 1:1000 or higher dilutions of the polysaccharides with type 2 pneumococcus antiserum; +, +, +, precipitation in tests of 1:10 million and lower dilutions of the polysaccharide.

The *Neisseria* polysaccharides, both of enzymatic and of living culture origins, differed from corn amylose in nearly all of the properties listed in Table IV; furthermore, the magnitude of the differences, especially in the iodine reactions, was sufficient to show that little or no amylose was present as an accompanying substance in either of the bacterial products.

The sharp distinction between the *Neisseria* polysaccharides and the dextran of *Leuconostoc mesenteroides* is of special interest because they are derived from the same substrate (sucrose). The dextran failed to show any hydrolysis by the amylases, failed to give any of the reactions with iodine, and was hydrolyzed by acid at a rate one-fourth to one-fifth that of the *Neisseria* products. That dextran was not contained, even in trace amounts, in either *Neisseria* polysaccharide was shown by the failure of the *Neisseria* products to give serological precipitation in dilutions as low as 1:1000 with an antiserum capable of revealing the presence of dextran in dilutions as high as 1:10 million.

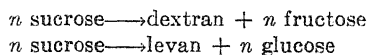
#### DISCUSSION

Glycogen-like polysaccharides were synthesized from sucrose and from glucose-1-phosphate (and apparently also from amylose<sup>2</sup>) through the action of soluble enzymes obtained from cultures of *Neisseria perflava*. We believe that this is the first instance in which the conversion of sucrose to polysaccharide material of the starch-glycogen class has been demonstrated in a system free of living cells.

The data on the nature and quantities of the products formed from sucrose established that the over-all reaction consists of the conversion of  $n$  molecules of sucrose to a glycogen-like polymer of  $n$  (glucose —  $\text{H}_2\text{O}$ ) residues plus  $n$  molecules of free fructose, apparently by direct polycondensation. The fact that glucose-1-phosphate also served as a substrate raised the possibility that that phosphorylated sugar might be required as an intermediate substance in the synthesis of the polysaccharide from sucrose. However, only very small amounts of phosphorus were present in active enzyme-sucrose systems, and no detectable change in the phosphorus partition occurred during polysaccharide synthesis from sucrose; moreover, the presence of inorganic phosphate in concentrations sufficiently high (8 moles of phosphate to 1 of substrate) to prevent all synthesis from glucose-1-phosphate had no inhibitory effect whatsoever upon the synthesis from sucrose. Furthermore, the activity for sucrose and the activity for glucose-1-phosphate were at least partially separated by mild heating treatment and by gas treatment. Thus, the evidence as a whole indicates strongly that the *Neisseria* extracts contain two systems: an *amylosucrase*, active preferentially if not solely upon sucrose, and a *phosphorylase*, active upon glucose-1-phosphate but not upon sucrose; and that, when the

amylosucrase acts upon sucrose, the synthesis of polysaccharide of the starch-glycogen class proceeds by a pathway that does not involve glucose-1-phosphate. This mechanism is contrary to the general belief (expressed, for example, by Cori (22) and by Hassid, Doudoroff, and Barker (23)) that all members of the starch or glycogen class are always formed from glucose-1-phosphate through the action of phosphorylases.

The syntheses of dextrans and levans from sucrose by enzymes from *Leuconostoc mesenteroides* (24-26) and *Aerobacter levanicum* (27, 28) also apparently proceed without mediation of glucose-1-phosphate or of any other phosphorylated sugar, according to the equations:



We have suggested the name *amylosucrase* for the enzyme system of *Neisseria* that converts sucrose to glycogen- or amylopectin-like material in order to indicate its close relationship to dextranucrase and levansucrase, as well as to emphasize its distinction from the phosphorylase plus cross-linking enzyme systems which bring about the synthesis of amylopectins and glycogens from glucose-1-phosphate (1, 29). However, in spite of the apparent differences, the enzymatic syntheses of polysaccharides from sucrose and from glucose-1-phosphate are fundamentally much alike in that the substrate in each instance contains the basic unit of the final polymer product in the form of a glycoside radical that is exceedingly easily split off by acids. This point of similarity was first observed (25) in a comparison of dextran and levan formation by bacterial enzymes with starch formation by plant and animal phosphorylases, in which instances the polysaccharide products were different. The similarity is even more prominent in the present example in which the product formed from sucrose by *Neisseria* amylosucrase is essentially like the glycogen formed from glucose-1-phosphate by tissue phosphorylases. The activity of glucose-1-phosphate as a polysaccharide precursor thus would appear to depend not upon its being a phosphate "ester," as is commonly believed, but upon a structural feature which is possessed also by the sucrose molecule (*cf.* (29)). Sucrose not only enters enzymatic reactions in which its glucoside and fructoside units are transferred to polysaccharides of several types (dextrans, levans, glycogen-like polysaccharides), but also enters reactions in which its glucoside group is transferred to glucose-1-phosphate and to several disaccharides (30, 31). This versatility in capacity to donate glycoside groups, though so far observed only with bacterial enzymes, deserves recognition as one of the most important biochemical properties of sucrose.

## SUMMARY

Cell-free extracts derived from *Neisseria perflava*, a variety of bacteria that occurs commonly in human throats, synthesized a glycogen-like polysaccharide from sucrose. The extracts also acted upon glucose-1-phosphate, but the actions on the two substrates could be distinguished on the basis of differences in the influence of high concentrations of inorganic phosphate and in the susceptibilities to heat and to gas treatment. The *Neisseria* extracts apparently contained a phosphorylase system, active upon glucose-1-phosphate but not upon sucrose, in addition to a new, heat-labile, enzyme system (*amylosucrase*) which has the capacity of forming polysaccharide material of the starch-glycogen class from sucrose by a pathway (direct polycondensation) that does not involve glucose-1-phosphate.

The polysaccharide formed by the *Neisseria* enzymes was more like glycogen than like the amylopectin formed by the living *Neisseria perflava* cultures or the amylopectin component of plant starch; it was much different from the dextran which *Leuconostoc mesenteroides* forms from sucrose.

The *in vitro* conversion of sucrose to maltose was accomplished by the successive action of *Neisseria* enzymes and of  $\beta$ -amylase.

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# COMPOSITION OF THE STARCH SYNTHESIZED FROM GLUCOSE-1-PHOSPHATE BY DIPHTHERIA BACILLI\*

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(Received for publication, July 28, 1948)

We have recently shown (1) that the diphtheria bacillus, *Corynebacterium diphtheriae*, is able to synthesize a starch-like product from glucose-1-phosphate; glucose or sucrose did not serve as substrates for the reaction. A similar capacity is also possessed by certain streptococci and by *Neisseria perflava* (1, 2), but it apparently is not common among other bacteria and thus may possibly be a feature useful for the description or identification of the diphtheria bacillus. The results of a detailed investigation of the occurrence of the starch-producing capacity among pathogenic and non-pathogenic members of the *Corynebacterium* genus, as well as of the general mechanism of the reaction, will be reported in another paper.<sup>1</sup>

The present experiments deal with the chemical nature of the product formed from glucose-1-phosphate by suspensions of *Corynebacterium diphtheriae* cells. Separation was accomplished by the method devised by Schoch (3) for the fractionation of plant starches; and, in the studies made on the properties of the isolated fractions, purified amylopectin and amylose fractions from corn-starch, kindly furnished by Dr. Schoch, were included along with mammalian glycogen in order to be able to relate the product of the diphtheria bacillus to polysaccharides of the starch-glycogen class of plant or animal origin.

## EXPERIMENTAL

### *Isolation of Polysaccharides Formed from Glucose-1-phosphate by Diphtheria Bacilli*

A recently isolated, virulent strain of *Corynebacterium diphtheriae* (mitis type) was utilized. Young cells were obtained by centrifugation of 15 liters of broth (1 per cent tryptose, 0.3 per cent yeast extract, 0.2 per cent glucose, and 0.2 per cent  $K_2HPO_4$ ) which had been heavily seeded and incubated at 37° for 18 hours. The washed cells, which contained only a small amount (0.09 gm.) of polysaccharide material, were added to 300 ml. of a 0.05 M glucose-1-phosphate solution, buffered at pH 6.4 by 0.1 M

\* Aided by a grant from the Sugar Research Foundation, Inc.

<sup>1</sup> Hehre, E. J., Carlson, A. S., and Neill, J. M., unpublished manuscript.

maleate. The mixture was incubated at 25° for 42 hours, when the glucose-1-phosphate (4) and inorganic phosphate (5) contents indicated that 71 per cent of the original glucose-1-phosphate had been utilized; the polysaccharide content (6) had risen to 1.57 gm.

In order to remove the lipid substances which are abundant in diphtheria bacilli (7) and which are undesirable for the present method of starch fractionation (3), the mixture was adjusted to pH 7.0, treated with 1700 ml. of absolute methanol, refluxed (68°) for 45 minutes, and then centrifuged. The sediment (containing the bacterial cells and the polysaccharide) was extracted in 180 ml. of 1.0 N NaOH at 25° for 90 minutes; three successive extractions were required to bring approximately 80 per cent of the polysaccharide into solution.<sup>2</sup> The collected fluids were without delay brought to pH 6.6 with glacial acetic acid, and then heated at 93° for 15 minutes. Insoluble material containing a negligible amount of polysaccharide was removed by centrifugation, and the still hot fluid (600 ml.) brought to pH 6.0 and 93°, and treated with 60 ml. of pentasol.<sup>3</sup> The mixture, after having been cooled slowly with continuous stirring over a period of 13 hours, was found to contain large numbers of microscopic crystalline particles that were stained intensely blue with iodine. These were separated from an opalescent supernatant fluid by high speed centrifugation in the cold, redissolved in hot water, and recrystallized by the use of pentasol. The final precipitate or Fraction A, the crystalline appearance of which is illustrated in Fig. 1, was washed twice with methanol and dried *in vacuo* at 25° over CaCl<sub>2</sub>. The yield was 0.43 gm. (moisture-free basis), or 27 per cent of the polysaccharide contained in the cell-substrate mixture.

The opalescent fluid from the original pentasol treatment was adjusted to pH 5.0 and treated with 2 volumes of alcohol; the resulting precipitate, collected by centrifugation, was twice extracted with 200 ml. of cold 1 per cent sodium acetate. A residue of 0.25 gm. remained, less than half of which was polysaccharide of the starch class. The soluble material, Fraction B, was reprecipitated with 2 volumes of alcohol, washed with absolute alcohol, and dried *in vacuo*. The yield was 0.63 gm. (moisture-free basis), or 40 per cent of the polysaccharide in the original mixture.

<sup>2</sup> In addition to the soluble material there was a final residue of 0.60 gm. which consisted mainly of cellular debris. This residue, although it contained 0.28 gm. of polysaccharide and was stained blue-black with iodine, was discarded because the polysaccharide could be obtained only by drastic extraction procedures.

<sup>3</sup> Pentasol is a mixture of five isomeric amyl alcohols, manufactured by Sharples Chemicals, Inc., Wyandotte, Michigan, which was found by Schoch (3) to be a very effective fractionating agent for plant starches.

*Properties of Isolated Polysaccharide Fractions*

Fraction A was a white crystalline substance which was nearly insoluble in cold water but which dissolved in cold 1.0 *N* NaOH, while Fraction B was a white amorphous powder that proved to be only partially soluble in cold water, despite the fact that at one stage in its preparation it had been completely soluble. The polyglucoside nature of both Fraction A and Fraction B is evident from the results of chemical analyses which are summarized in Table I. That both fractions are members of the starch class was indicated by the color reactions given with iodine as well as by the high

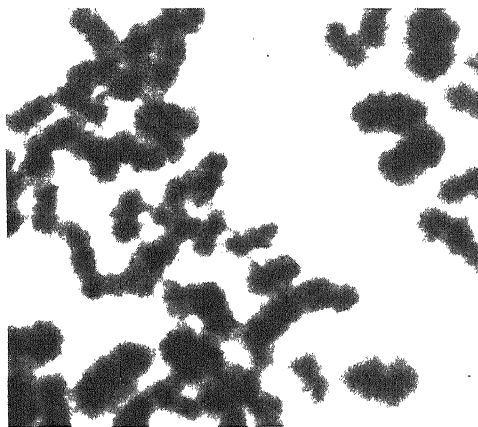


FIG. 1. Crystalline *Corynebacterium diphtheriae* Fraction A, prepared by pentasol treatment and stained by iodine; 1000  $\times$ .

positive optical rotations. However, in order to characterize the polysaccharides further a number of tests used for the differentiation of polysaccharides of the starch-glycogen class were made simultaneously on Fractions A and B, on purified amylopectin and recrystallized amylose fractions from corn-starch, and on a sample of glycogen from mammalian liver.

The extent of hydrolysis by  $\alpha$ - and  $\beta$ -amylases was determined with (1) a clarified 1:5 dilution of saliva in physiological salt solution; (2) 20 mg. of analytical grade  $\beta$ -amylase from ungerminated barley (furnished by Wallerstein Laboratories, New York); and (3) 2 mg. of  $\beta$ -amylase prepared from whole wheat flour (12). A sample of 8 to 10 mg. of polysaccharide was dissolved in 2.0 ml. of cold 1.0 *N* NaOH, and adjusted to pH 5.8 for the  $\alpha$ -amylase and to pH 4.6 for the  $\beta$ -amylase with 1.0 *N* acetic acid; 1.0 ml. of enzyme solution was immediately added, and the mixture

diluted to 10.0 ml. with water. After incubation at 30° for 24 hours ( $\alpha$ -amylase) or 2 days ( $\beta$ -amylase), the reducing sugar content was determined (10) and calculated as maltose according to the data of Weise and Brand (13). The values are reported in terms of percentage of the maltose that theoretically would be obtainable after correction for the entry of water during the hydrolysis.

The "blue value" with iodine was determined by a slight modification of the method of McCready and Hassid (14). Alkaline solutions, containing 1.0 mg. of polysaccharide per ml., were prepared as directed (14); 5.0 ml. were diluted with 50 ml. of water, and 2.7 ml. of 0.1 N acetic acid

TABLE I

*Analysis of Fractions Isolated from Incubated Mixture of Corynebacterium diphtheriae Cells and Glucose-1-phosphate\**

Fraction	Color with iodine	Ash, as Na <sub>2</sub> SO <sub>4</sub>	Reducing power (8)	Nitrogen (9)	Phosphorus (5)	[ $\alpha$ ] <sub>5893</sub> <sup>22†</sup>	Acid hydrolysate‡	
							[ $\alpha$ ] <sub>5893</sub> <sup>22</sup>	Reducing sugar (10), as glucose§
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>degrees</i>	<i>degrees</i>	<i>per cent</i>
A	Intense blue	0.0	<0.1	0.1	0.01	+164 ( <i>c</i> = 0.9)	+49.9 ( <i>c</i> = 0.6)	93.3
B	Purple	1.2	0.4	0.45	0.16	+165 ( <i>c</i> = 1.0)	+47.5 ( <i>c</i> = 0.6)	90.5

\* Calculations are on the ash- and moisture-free basis.

† The optical rotations were determined on solutions in 0.5 N NaOH.

‡ The polysaccharides were hydrolyzed for 2.5 hours in 1.0 N HCl. The data are corrected for the entry of water.

§ Abundant glucosazone (m.p. 205–206°, uncorrected) was obtained when the hydrolysates were treated with phenylhydrazine; the absence of pentoses and uronic acids was indicated by the negative qualitative Bial test (11).

|| Per cent of the reducing power of glucose.

added to bring the reaction to pH 5.8; after mixing, 2.5 ml. of 0.2 per cent iodine and 2 per cent KI were added, and the volume brought to 250 ml. with water. The intensity of the blue color was measured by the use of a Klett-Summerson colorimeter (test-tube model) with red Filter 66. The results are expressed as scale readings, as in the paper of McCready and Hassid (14). The degree of complex formation with iodine was measured by Schoch's modification (15) of the potentiometric method of Bates, French, and Rundle (16). One-fourth the prescribed weight of polysaccharide was used, but since the recommended concentrations of polysaccharide and of all reagents were maintained, our procedure was strictly comparable to that of Schoch (15). The results are expressed as per cent iodine bound in complex formation (mg. of I<sub>2</sub> per 100 mg. of dry weight of polysaccharide).

Finally the products were tested for their capacity to precipitate with iodine, pentasol, and butanol. For the test with iodine, 1.0 ml. of a solution containing 0.02 per cent  $I_2$  and 0.8 per cent KI was added to 2.0 ml. of 0.05 per cent polysaccharide in acetate buffer at pH 4.4; the mixtures were observed for precipitation at intervals from 15 minutes to 1 week. For the pentasol and the butanol tests, 0.5 ml. of the alcohol was added to 2.0 ml. of a 0.5 per cent solution of polysaccharide in acetate buffer (pH 6.2)

TABLE II

*Properties of Corynebacterium diphtheriae Polysaccharides in Comparison with Those of Corn-Starch Fractions and of Glycogen\**

Source and kind of polysaccharide	Con- version to mal- tose by $\alpha$ -amy- lase	Conversion to maltose by $\beta$ -amy- lase		Iodine bound in complex forma- tion†	Blue value with iodine	Ppt. with various reagents		
		Barley	Wheat			Iodine‡	Pentasol	Butanol
	per cent	per cent	per cent	per cent				
<i>C. diphtheriae</i> , Frac- tion A	97	86	85	17.2	278	++	Crystalline	Amorphous
Corn amylose (Schoch)	101	91	94	18.9§	298	++	"	Crystalline
<i>C. diphtheriae</i> , Frac- tion B	87	67	64	5.7	77	+	0	0
Corn amylopectin (Schoch)	92	64	60	0.6§	32	0	0	0
Rabbit liver glycogen	73	43	39	<0.1	0	0	0	0

\* All the data are calculated to the moisture- and ash-free basis.

† The derivation of these values is shown in Fig. 2.

‡ ++, copious blue precipitate in 15 minutes; +, violet precipitate after 1 day; 0, no precipitate after 1 week.

§ Dr. Schoch (personal communication) found iodine affinity values of 18.6 per cent for the sample of corn amylose and 0.7 per cent for the corn amylopectin.

that had been heated to 95°; the mixture was permitted to cool to 25° over a period of 24 hours, then centrifuged, and observed for the occurrence and the microscopic nature of the sediment.

It is evident from the data in Table II that Fraction A and Fraction B, synthesized from glucose-1-phosphate by *Corynebacterium diphtheriae*, resembled in a general way the two major components of plant starches.

Fraction A appears to be a polysaccharide of the amylose class. In the degree of conversion by  $\alpha$ - and  $\beta$ -amylases, in the "blue value," and in the amount of complex formation with iodine, Fraction A gave values between 90 and 96 per cent of those obtained with corn amylose. It was like the corn product also in respect to rapid and complete precipitation by excess iodine, and, as shown in Fig. 2, had a closely similar iodine adsorption

curve. Pentasol produced a crystalline precipitate with both; and the crystals of Fraction A (Fig. 1), although somewhat smaller and less sharply defined, were similar in appearance to those found with corn amylose. On the other hand, treatment with butanol, which gave a crystalline precipitate with the corn amylose, caused only the formation of an amorphous precipitate with Fraction A.

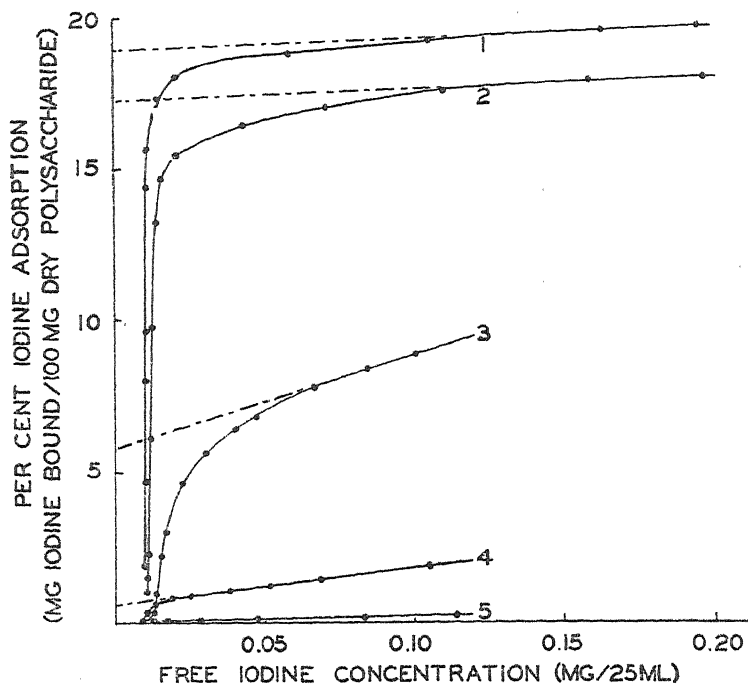


Fig. 2. Curves, derived from potentiometric measurements, showing the iodine adsorption of the *Corynebacterium diphtheriae* and reference polysaccharides as a function of free iodine concentration. Curve 1, corn amylose, Curve 2, *C. diphtheriae* Fraction A, Curve 3, *C. diphtheriae* Fraction B, Curve 4, corn amylopectin, Curve 5, rabbit liver glycogen. The point of interception with the ordinate, made by the dotted extension of each curve, was taken as the amount of iodine bound by the polysaccharide in complex formation.

Fraction B behaved like corn amylopectin in respect to the extent of its hydrolysis by  $\alpha$ - and  $\beta$ -amylases, and in its failure to be precipitated from dilute solution by pentasol or butanol. However, the iodine reactions and solubility characteristics of Fraction B were intermediate between those of corn amylopectin and those of corn amylose or Fraction A. That is, the "blue value" of Fraction B was somewhat greater than that of the amylopectin, and the degree of complex formation as well as the entire

curve for iodine adsorption was intermediate between those of the plant fractions (Fig. 2); moreover, Fraction B showed delayed precipitation with iodine, was only partially soluble in cold water, and had a tendency to precipitate from solution in the cold. Fraction B may thus be considered to be a unique amylopectin-like polysaccharide, some of whose properties are intermediate between those of amylopectin and amylose; that it is not an amylose whose properties were being masked by adsorbed lipid material was shown by tests made after exhaustive extraction with 85 per cent methanol.

#### DISCUSSION

Although a portion of the starch-like product synthesized from glucose-1-phosphate by suspensions of *Corynebacterium diphtheriae* was not examined because of the difficulty of its extraction from the bacterial cells, the major part of the synthetic polysaccharide was separated and fractionated by the method of Schoch (3) into two components. One of these (Fraction A) was obtained in the form of microscopic crystals; it had properties that closely resembled those of corn amylose, and it can in fact be regarded as an amylose. The other (Fraction B) proved to be an unusual member of the starch class; some of its properties were like those of corn amylopectin, while others were intermediate between those of the amylopectin and amylose of corn-starch. The amounts of Fractions A and B isolated accounted for 27 and 40 per cent respectively of the total polysaccharide formed by the mixture of *Corynebacterium diphtheriae* cells plus glucose-1-phosphate.

The *Corynebacterium diphtheriae* product as a whole appears to be similar in a general way to the common root and cereal starches, which comprise two components. It is true that the ratio of Fraction A to Fraction B is somewhat higher than that of amylose to amylopectin in these plant starches, and that the qualitative properties of Fraction B of the *C. diphtheriae* starch do not entirely correspond to those of the amylopectins of the plant starches. Nevertheless, the product formed from glucose-1-phosphate by the diphtheria bacillus does approach those natural starches which consist of mixtures of linear (amylose) and branched (amylopectin) fractions more closely than do the "synthetic starches" formed from glucose-1-phosphate by phosphorylases, which consist essentially only of amylose (17, 18). Since the *C. diphtheriae* product contains an apparently branched component, its formation must involve, in addition to phosphorylase, some other factor like the cross-linking enzymes occurring in liver and potatoes (19, 20) which bring about the formation of glycogen and amylopectin.

The *Corynebacterium diphtheriae* amylose component is of some bio-

chemical interest as the first example of the crystallization by the method of Schoch (3) of a product definitely known to have been synthesized from glucose-1-phosphate. Moreover, with the exception of the low molecular weight Schardinger dextrins which have long been known to crystallize from the cultures of certain bacilli (21), the *Corynebacterium diphtheriae* amylose represents the first bacterial polysaccharide to be obtained in crystalline form.

#### SUMMARY

The starch-like material synthesized by diphtheria bacilli from glucose-1-phosphate was studied with the aid of the fractionation method of Schoch, through direct comparison with several reference polysaccharides of the starch-glycogen class from plant and animal sources. The *Corynebacterium diphtheriae* product was a mixture of two components, as in the case of the common root and cereal starches. One of the components, which was an amylose similar to the amylose components of the plant starches, was obtained in crystalline form. The other component was a unique amylopectin-like polysaccharide, some of whose properties were intermediate between those of the amylopectin and amylose fractions of plant starch.

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# CRYSTALLINE AMYLOSE FROM CULTURES OF A PATHOGENIC YEAST (*TORULA HISTOLYTICA*)\*

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(Received for publication, July 28, 1948)

Mager and Aschner (1, 2) have reported that certain yeasts belonging to the genus *Torulopsis*, when grown under the proper conditions, produce an extracellular polysaccharide that gives an intense blue color with iodine, and have suggested (3) a method for the identification of the pathogenic species *Torulopsis neoformans* (i.e., *Torula histolytica*) based in part on that capacity. The blue-staining product was originally thought to correspond to the amylose component of the starch of green plants (1), but more recently Mager (4) has reported that the product formed by the saprophytic *Torulopsis rotundata* differs in chemical properties both from the amylose and from the amylopectin components of starch. The analyses of Mager (4), however, are difficult to interpret because they were made chiefly on material that was only about 26 per cent pure.

The present experiments deal with the isolation and chemical properties of the blue-staining material from a culture of *Torula histolytica*. For the isolation the method of Schoch (5) was employed because preliminary trials showed it well suited for the separation of the relatively small amount of the desired product from the large amount of other polysaccharide accompanying it in the *Torula* culture. In order to determine the position of the *Torula* product in the starch group of polysaccharides, the chemical study included comparisons with fractions of corn-starch and with glycogen.

## EXPERIMENTAL

*Separation of Torula Polysaccharides*—The strain of *Torula histolytica* used had been isolated from the spinal fluid of a patient with fatal meningo-encephalitis; it was obtained through the courtesy of Miss Mary E. Hopper of the New York Hospital. This strain did not have prominent capsules when cultivated *in vitro*, but it was pathogenic for mice and produced large capsules *in vivo*. When grown under the conditions described by Mager and Aschner (3), extracellular material that was stained intensely blue with iodine was produced.

In order to obtain the blue-staining polysaccharide for analysis, 10 liters of the Mager and Aschner medium (1 per cent glucose, 0.1 per cent

\* Aided by a grant from the Sugar Research Foundation, Inc.

( $\text{NH}_4$ ) $_2$ SO $_4$ , 0.1 per cent KH $_2$ PO $_4$ , 0.05 per cent MgSO $_4$ ·7H $_2$ O, and 0.002 mg. per cent thiamine hydrochloride) were heavily inoculated and incubated at 27° for 5 days with continuous and vigorous aeration. The grown culture was adjusted to pH 7.0 with NaOH and then heated between 70–80° for 30 minutes in order to kill the microorganisms. The heated culture was rapidly cooled and treated with 2.5 volumes of alcohol, and the bulky precipitate of yeast gum and cells was centrifuged immediately. The soluble constituents of this sediment were obtained by three successive extractions with a total of 1 liter of 0.02 M acetate buffer, pH 6.0. For each extraction the sediment was suspended in buffer, heated at 93° for 15 minutes, and centrifuged. The final residue of yeast cells and cellular debris (26.8 gm. when dried) gave only a maroon color with iodine and was not further studied. The combined extraction fluids, which gave an intense blue color with iodine at a dilution of 1:50, were again brought to 93°; 80 ml. of pentasol<sup>1</sup> were added and the mixture cooled to 30°, with continuous stirring, over a period of 12 hours. Large numbers of crystalline particles which were stained blue with iodine were evident on microscopic examination. The crystals were separated from the highly opalescent supernatant fluid by high speed centrifugation, and recrystallized without appreciable loss by a second treatment with pentasol. The final material was washed twice with methanol and dried *in vacuo* at 25° over CaCl $_2$ . The pure white product, Fraction A, weighed 0.16 gm., which proved to be less than one-thirtieth of the water-soluble material obtained from the culture.

The bulk of the extracellular material produced by the *Torula* was in the opalescent supernatant fluid that remained after separation of the above crystalline precipitate. This fluid, which gave some red but no blue color with iodine, when treated with 2.5 volumes of alcohol, yielded a precipitate of 4.62 gm. dry weight. The study of this material which we call Fraction B has not yet been completed, but evidence has been obtained that it is a mixture of several components, including (1) a glycogen-like material and (2) a capsular substance, not of the starch class but apparently a pentose-containing polysaccharide, which Dr. James M. Neill<sup>2</sup> has found capable of giving serological precipitation in high dilutions with appropriate anti-serums.

*Properties of Fraction A*—Although nearly insoluble in water, Fraction A dissolved readily in cold 1.0 N NaOH. It showed an unusually pronounced tendency to precipitate from neutralized solutions, whether kept warm or

<sup>1</sup> Pentasol is a mixture of five isomeric amyl alcohols manufactured by Sharples Chemicals, Inc., Wyandotte, Michigan.

<sup>2</sup> Neill, J. M., Castillo, C. G., Smith, R. H., and Kapros, C. E., *J. Exp. Med.*, in press.

cold. Its crystalline appearance (Fig. 1) closely resembled a sheaf of needles and was like that of pentasol-precipitated corn and potato amyloses. However, in spite of its crystallinity, the *Torula* product was not chemically pure.<sup>3</sup> Analyses showed an ash content (as  $\text{Na}_2\text{SO}_4$ ) of 0.5 per cent, phosphorus (6) 0.03 per cent, and a nitrogen content (7) of 0.74 per cent; on the basis of the latter value, we have assumed the presence of a "protein" impurity of 4.6 per cent. Correction for all these impurities as well as for moisture has been applied to the following analytical data.

The rotation before hydrolysis was  $[\alpha]_{5893}^{22} = +142^\circ$  ( $c = 0.5$  in 0.5 N NaOH). After hydrolysis in 1.0 N HCl for 2.5 hours at  $100^\circ$ , with correc-

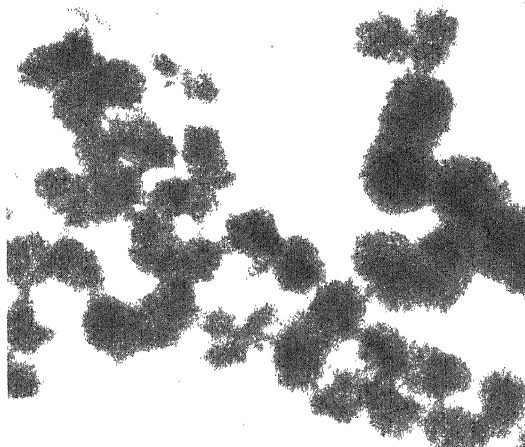


FIG. 1. Crystalline amylose (Fraction A) from *Torula histolytica*, stained with iodine;  $1000\times$ .

tion for the entry of water, the rotation was  $[\alpha]_{5893}^{22} = +43^\circ$  ( $c = 0.4$ ) and the reducing sugar content 91 per cent as glucose (8). The hydrolysate

<sup>3</sup> In addition to the impurities indicated by the chemical analyses, Fraction A also contained some small amount of material which gave precipitation with the same antisera that reacted with the capsular polysaccharide included in Fraction B. If, as we believe, this material represents traces of the capsular polysaccharide, the amount present in Fraction A may be roughly estimated to be about 1 per cent; that estimation is made on the basis that, in parallel tests against the same antiserum, Fraction A gave precipitation in dilutions of 1:5000 to 1:10000 but not at higher dilutions, whereas the purified capsular polysaccharide gave positive reactions when diluted 1:1 million or 1:2 million. It is worth while to note that serological tests have certain advantages in the detection of traces of polysaccharides contained as accompanying substances in other carbohydrate materials, as exemplified by the serological detection of the presence of dextran in reagent sucrose of the highest degree of chemical purity (10).

yielded glucose phenylosazone, with a melting point of  $206^{\circ}$ , when treated with phenylhydrazine. A qualitative (Bial) test (9) for pentoses and uronic acids was negative; this test was sufficiently sensitive to have detected pentose if present to the extent of more than 1 per cent of the reducing sugar of the hydrolysate.

The *Torula* polysaccharide was further examined, along with several reference polysaccharides of the starch-glycogen class, for the extent of conversion to maltose by  $\alpha$ -amylase from saliva and by  $\beta$ -amylase from ungerminated barley, the degree of complex formation with iodine, as determined potentiometrically, the intensity of blue color ("blue value"), and the capacity to be precipitated by pentasol and by iodine. These determinations were made in the manner described in a preceding paper

TABLE I

*Properties of Crystalline Torula Polysaccharide in Comparison with Those of Corn-Starch Fractions and of Glycogen*

Source and kind of polysaccharide	Conversion to maltose by $\alpha$ -amylase	Conversion to maltose by $\beta$ -amylase	Iodine bound in complex formation	Blue value with iodine	Ppt. with iodine*	Ppt. with pentasol
	per cent	per cent	per cent†			
<i>T. histolytica</i> Fraction A. . . . .	93‡	86‡	19.5	356	++	Crystalline
Corn amylose (Schoch) . . . . .	101	92	18.9	315	++	"
" amylopectin (Schoch) . . . . .	92	60	0.6	32	0	0
Rabbit liver glycogen . . . . .	73	40	<0.1	0	0	0

\* ++, copious blue precipitate in 15 minutes; 0, no precipitate after 1 week.

† Mg. of iodine bound per 100 mg. of dry polysaccharide.

‡ Precipitation of some of the polysaccharide from solution occurred during the period of incubation with the enzyme; so that these values probably do not represent the maximal convertibility to maltose.

(11). The reference polysaccharides were samples of amylopectin and of crystalline amylose prepared by the pentasol method (5) from corn-starch, and a sample of glycogen from mammalian liver. All procedures were performed simultaneously on *Torula histolytica* Fraction A and on the reference materials. The results are summarized in Table I.

It is evident that the crystalline *Torula* polysaccharide had the properties of an amylose. The extent of its conversion by  $\alpha$ - and  $\beta$ -amylases approached those found with corn amylose, despite the precipitation from solution of some of the polysaccharide during the course of the digestion. The intensity of color with iodine, expressed as the "blue value," was higher for the *Torula* polysaccharide than for the corn amylose, while the amount of iodine adsorbed in complex formation by the *Torula* product

was approximately the same as that of the corn amylose. (Though not illustrated, the potentiometric curves from which the latter values were derived were closely similar.) Furthermore, in its precipitation by pentasol and by iodine, the *Torula* polysaccharide behaved like the linear corn fraction. The distinction between *Torula* Fraction A and both corn amylopectin and liver glycogen was absolute.

#### SUMMARY

The substance responsible for the intensely blue coloration with iodine given by cultures of the pathogenic yeast-like fungus *Torula histolytica* has been isolated in crystalline form and identified as a polysaccharide of the amylose class. Separation was achieved by application of the procedure developed by Schoch for the fractionation of plant starches, and the analyses made in comparison with specimens of plant amylose and amylopectin and of liver glycogen.

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# EXCRETION OF RADIOACTIVE CARBON DIOXIDE BY RATS AFTER ADMINISTRATION OF ISOTOPIC BICARBONATE, ACETATE, AND SUCCINATE\*

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(Received for publication, September 4, 1948)

The rate of excretion of  $C^{14}O_2$  has been followed after the intraperitoneal injection into rats of (1) isotopic bicarbonate,  $NaHC^{14}O_3$ , (2) isotopic acetate,  $CH_3C^{14}OONa$ , and (3) isotopic succinate,  $NaOOCCH_2CH_2C^{14}OONa$ . The data so obtained yield information on the rate at which  $CO_2$  participates in the metabolic processes of the body, as well as the rate at which isotopic carbon is eliminated following its administration in the three chemical forms.

Previous measurements of excretion of isotopic  $CO_2$  reported in the literature have been few and, in these few, the experiments were not designed to determine the rates of  $CO_2$  excretion following the administration of a single dose of isotopic material (1-3). In the present experiments, the excretion of  $C^{14}O_2$  and total  $CO_2$  has been measured at frequent intervals up to 4 hours.

## EXPERIMENTAL

White rats weighing 120 to 140 gm. were fasted for 24 hours prior to the experiment, given the isotopic material in about 1 ml. of solution by intraperitoneal injection, and placed in a metabolism chamber for collection of respiratory  $CO_2$ .

*Isotopic Solutions Used*—Isotopic  $NaHC^{14}O_3$  was prepared from a solution of isotopic  $Na_2C^{14}O_3$  diluted with M/15 phosphate buffer, pH = 7.4. The final volume was approximately 1 ml. An aliquot of 35 c.mm. was removed for determination of the radioactivity of the solution and the remainder was injected intraperitoneally into the rat. The total radioactivity injected amounted to 2.5 to  $5 \times 10^5$  counts per minute per rat. The total amount of  $CO_2$  injected was 0.025 to 0.05 mm.

Isotopic  $CH_3C^{14}OOH$  was prepared<sup>1</sup> according to the method of Sakami, Evans, and Gurin (4). The acetate solutions injected intraperitoneally

\* This work was supported in part by a contract between Harvard University and the Office of Naval Research, and in part by a grant-in-aid from Swift and Company.

<sup>1</sup> We are indebted to Dr. Yale J. Topper for the preparation of the isotopic acetate and succinate.

contained from  $0.6$  to  $3.2 \times 10^5$  counts per minute. In three experiments, the total acetate injected varied from  $0.01$  to  $0.02$  millimole of acetate dissolved in  $1$  ml. of isotonic NaCl solution. In a fourth experiment, carrier acetate was added, making the total acetate injected  $0.13$  mm.

Isotopic succinic acid labeled in the carboxyl group was prepared<sup>1</sup> as described in the accompanying paper (5). The amount injected intraperitoneally was  $0.8$  to  $1.5$  ml. of a solution containing  $0.1$  to  $0.5$  millimole of succinate and a total activity of  $0.8$  to  $2.5 \times 10^5$  counts per minute. These solutions were prepared by dissolving the succinic acid in an equivalent amount of NaOH, adjusting the pH to  $7.4$  with  $M/15$  phosphate, and bringing to volume.

*Measurement of CO<sub>2</sub> Excretion*—Immediately after injection of the solution containing isotopic carbon, the rat was placed in a simple metabolism chamber consisting of a large glass tube (inside diameter  $60$  mm., length  $300$  mm.), closed with one hole stoppers at each end, and collection of expired CO<sub>2</sub> was begun. A current of CO<sub>2</sub>-free air was drawn through the tube at the rate of about  $200$  to  $300$  ml. per minute. The expired CO<sub>2</sub> was collected by aspiration through  $100$  ml. of dilute NaOH contained in a glass column ( $4 \times 20$  cm.) sealed at the bottom to a sintered glass funnel. The amount of NaOH used was determined by the length of time the CO<sub>2</sub> was to be collected.  $2$  milliequivalents of NaOH were used for each minute that expired CO<sub>2</sub> was collected, thereby providing a  $20$ -fold excess of alkali.

Before admitting the air to the metabolism chamber, it was rendered CO<sub>2</sub>-free by passage through two towers filled with moistened soda lime. Tests without the rat revealed no detectable CO<sub>2</sub> in the incoming air. A trap containing Ba(OH)<sub>2</sub> was placed after the NaOH absorption column as a means of detecting incomplete absorption of CO<sub>2</sub> by the NaOH.

For the first  $30$  minutes, samples were collected at  $10$  minute intervals; thereafter, at  $30$  minute intervals for the duration of the experiment, which was usually about  $4$  hours. The NaOH solutions, containing the expired CO<sub>2</sub> as Na<sub>2</sub>CO<sub>3</sub>, were quantitatively transferred to volumetric flasks and made up to  $200$  ml. After determination of the CO<sub>2</sub> content by the Van Slyke manometric method, an aliquot of solution calculated to yield  $5$  mg. of BaCO<sub>3</sub> was treated with Ba(OH)<sub>2</sub>. The precipitate of BaCO<sub>3</sub> was washed and transferred to a stainless steel cup for radioassay with a thin window counter, and the radioassay results expressed as counts per minute.

### Results

The significant data in each experiment consisted of the amount of C<sup>14</sup> injected at zero time and the amount of C<sup>14</sup> and the total CO<sub>2</sub> excreted in each sample. The amount of C<sup>14</sup> per millimole of total CO<sub>2</sub> is the specific activity of the expired C<sup>14</sup>O<sub>2</sub>. The specific activities, adjusted to a uniform



initial injection of 100,000 counts per minute, have been plotted logarithmically against time in Fig. 1.

The data obtained in three bicarbonate experiments are individually shown and a smooth curve drawn through the points. The curve is linear for about 1 hour with a slope indicating a 50 per cent reduction in specific activity every 15 minutes. The decrease in specific activity during this period is interpreted as primarily a dilution of the isotopic  $\text{CO}_2$  of the body fluids with non-isotopic  $\text{CO}_2$  of metabolic processes.  $\text{CO}_2$  incorporation in organic reactions is doubtless also occurring during this period, but the

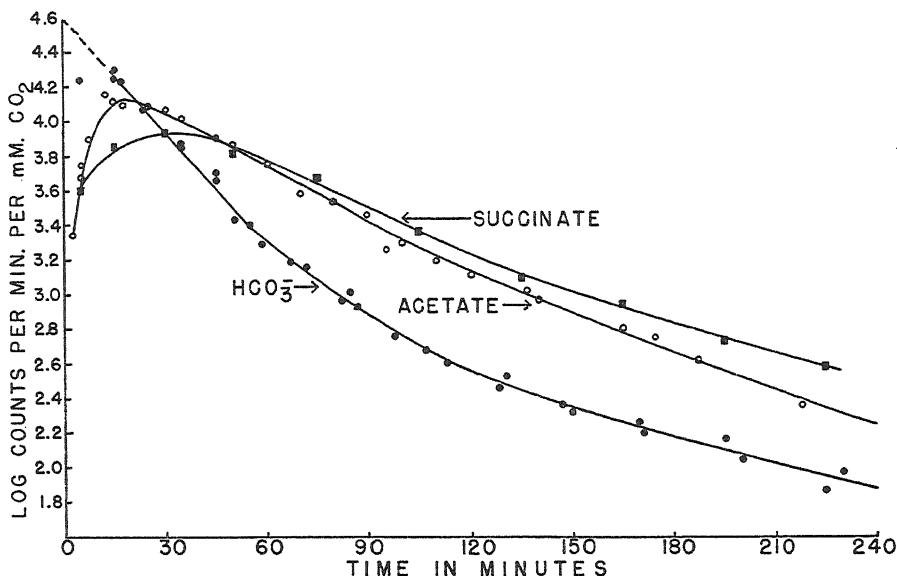


FIG. 1. The logarithm of the specific activity of  $\text{CO}_2$  excreted is plotted against time, following the intraperitoneal injection of isotopic sodium bicarbonate, acetate, and succinate.

effect of these reactions on the specific activity of the inorganic  $\text{C}^{14}\text{O}_2$  is small compared with the effect of dilution with non-isotopic  $\text{CO}_2$ .

After an hour, the rate of decrease in specific activity becomes progressively slower, requiring at the end of the experiment more than 100 minutes to produce a 50 per cent decrease in specific activity. This slowing is interpreted as denoting dilution of the isotopic inorganic carbon with both non-isotopic carbon and isotopic carbon returning to the inorganic carbon pool.

Extrapolation of the linear portion of the curve to zero time gives a log specific activity of 4.58 or a specific activity of 38,000 counts per minute per millimole of  $\text{CO}_2$ . Based on the injection of 100,000 counts per minute,

this would indicate that the  $C^{14}O_2$  initially injected was distributed in an inorganic carbon pool of 2.63 mm per 130 gm. rat, or 2 mm per 100 gm. rat. Since the total  $CO_2$  in the extracellular and intracellular fluids of a 100 gm. rat would amount to only about 1 mm of  $CO_2$ , the  $C^{14}O_2$  must have rapidly entered into mobile equilibrium with 1 mm of  $CO_2$  elsewhere in the body. One possibility is rapid exchange with the  $CO_2$  of bone, and another is incorporation into dicarboxylic and tricarboxylic acids. The present experiments do not permit a decision as to the relative importance of these two possibilities.

The data of the acetate experiments have been plotted as the average of three experiments. The curve drawn through the points shows a maximum specific activity of  $C^{14}O_2$  between 10 and 20 minutes, emphasizing the rapidity of the processes of acetate absorption, distribution, metabolism, and excretion of the  $C^{14}O_2$  formed. This curve is determined primarily by the balance between  $C^{14}O_2$  formed by oxidation and its dilution with non-isotopic  $CO_2$ .

During the period of 40 to 120 minutes, the decrease in the log specific activity was linear with a slope indicating a 50 per cent decrease in specific activity in 25 minutes. After this time, a slower rate of decrease was observed, becoming approximately parallel to the curve of the bicarbonate experiments during the later stages.

The data of the succinate experiments have been plotted as the average of three experiments. In the succinate experiments, the maximum specific activity of  $C^{14}O_2$  was reached at about 30 minutes. From 50 to 200 minutes, the curve was linear with a slope corresponding to 50 per cent decrease in specific activity in 35 minutes. The maximum specific activity was reached somewhat later than in the acetate experiments, but this may have been due to the larger amount of succinate injected and slower absorption from the peritoneal cavity.

*Cumulative Excretion of  $C^{14}O_2$* —The cumulative excretion of  $C^{14}O_2$  has also been calculated and the average values have been plotted for each series of experiments (Fig. 2). After 4 hours, the average total excretion of injected  $C^{14}$  amounted to 95 per cent after isotopic bicarbonate, 87 per cent after isotopic acetate, and 86 per cent after isotopic succinate. These final values varied in individual experiments by  $\pm 5$  per cent from the mean. Because of these variations, it is not to be concluded that the residual  $C^{14}$  in the body of the rat at the end of 4 hours is known with certainty in any one of the present experiments. However, the data show that 50 per cent of the  $C^{14}$  is excreted in 18 minutes when administered as  $NaHC^{14}O_3$ , whereas after  $C^{14}$  administration as carboxyl-labeled acetate and carboxyl-labeled succinate, 42 and 55 minutes, respectively, are required for 50 per cent  $C^{14}$  elimination.

*Radioactivity of Liver Constituents*—A few results were obtained on the distribution of isotopic carbon in glycogen, fatty acids, and cholesterol isolated from the livers of the rats at the end of the experiments. Following isotopic bicarbonate injection, the liver glycogen had appreciable amounts of  $C^{14}$  but no  $C^{14}$  was detected in the fatty acid or cholesterol fractions of the liver. On the other hand, following isotopic acetate injection, isotopic carbon was present in all three liver components. After the administration of labeled succinate, isotopic carbon was found only in glycogen and not in the fatty acids or cholesterol.

Since the amount of residual radioactive carbon in the body of the rats at the time of their sacrifice was very small, the failure to find measurable amounts of radioactivity in a tissue fraction is not to be interpreted as evidence that none was there.

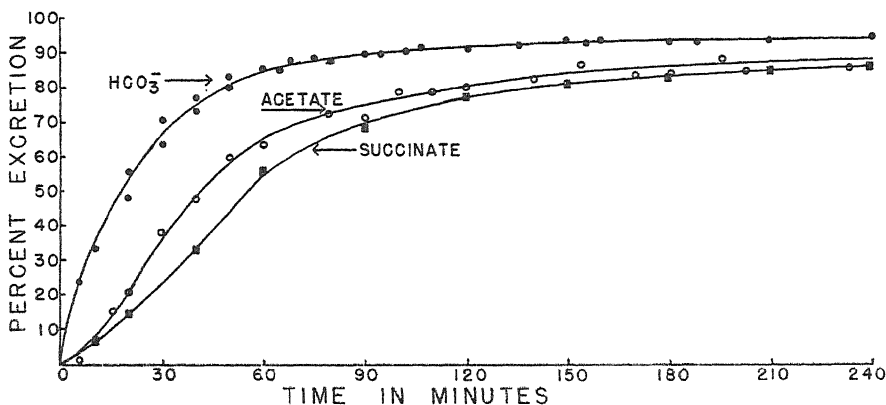
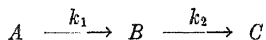


FIG. 2. The calculated cumulative  $C^{14}O_2$  excretion is plotted against time, following the administration of isotopic sodium bicarbonate, acetate, and succinate.

#### DISCUSSION

The cumulative recovery curves of  $C^{14}O_2$  excretion after the administration of labeled acetate and succinate (Fig. 2) do not show the rates of metabolism of the labeled materials without further analysis of the data.

However, such information may be obtained by calculating the distribution of the  $C^{14}$  in the organic and inorganic chemical species, in the system as a whole, at any one time. By "system as a whole" is meant the rat plus the total  $C^{14}O_2$  which has been excreted. The problem is treated as one involving two consecutive processes, (1) oxidation and (2) excretion, these being of the general form:



## RADIOACTIVE CARBON DIOXIDE EXCRETION

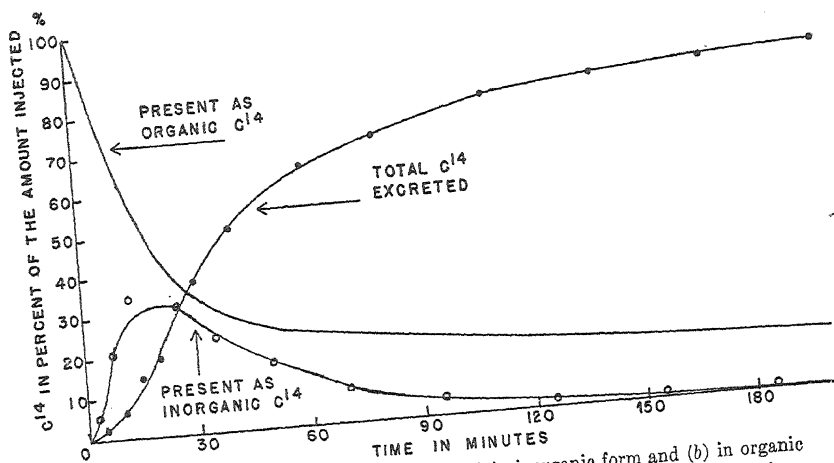


FIG. 3. The  $C^{14}$  remaining in the animal (a) in inorganic form and (b) in organic form, following the administration of isotopic sodium acetate, is plotted against time.

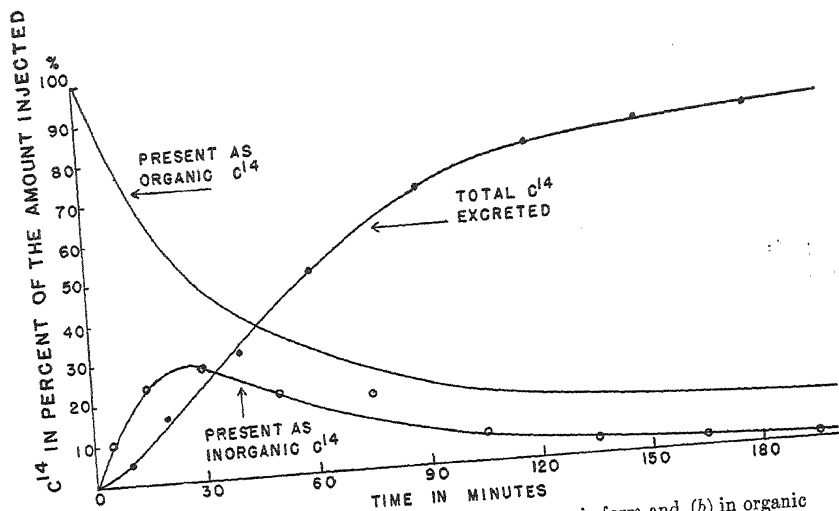


FIG. 4. The  $C^{14}$  remaining in the animal (a) in inorganic form and (b) in organic form, following the administration of isotopic sodium succinate, is plotted against time.

where  $k_1$  is the velocity constant of conversion of  $C^{14}$  in organic form to  $C^{14}O_2$ , and  $k_2$  is the velocity constant of excretion of  $C^{14}O_2$ .

From the experiments with labeled bicarbonate (Fig. 2), the excretion of  $C^{14}O_2$  appears to follow a first order reaction for about 60 minutes. This may be expressed by the equation

$$(C^{14}O_2)_t = 100e^{-k_2t}$$

where  $(C^{14}O_2)_t$  = the per cent of  $C^{14}O_2$  excreted in time,  $t$ , and  $k_2$  has the value of 0.047.

The values of  $k_1$  for the conversion of  $C^{14}$  in the carboxyl groups of acetate and succinate have been estimated from the calculated amount of  $C^{14}$  present in organic form (Figs. 3 and 4). (These values were obtained from the measurements of the total  $C^{14}O_2$  excreted and the calculated  $C^{14}O_2$  in the animal.) In the acetate experiments, the average value of  $k_1 = 0.043$ , and in the succinate experiments,  $k_1 = 0.028$ , thus showing that the rate of conversion of acetate carboxyl carbon to  $CO_2$  is about the same as the rate of excretion of  $CO_2$ , whereas the rate of conversion of succinate carboxyl carbon to  $CO_2$  appears to be slower.

The quantitative relations reached from the analysis of the data presented above must be regarded as tentative and subject to revision as further experiments are completed. They are presented at this time in order to illustrate the type of information which can be obtained from respiratory  $CO_2$  data following the administration of isotopic materials. It is believed that the method may find useful application in the study of abnormal metabolic states in the intact animal.

#### SUMMARY

1. The rates of excretion of  $C^{14}O_2$  by normal rats have been measured following the intraperitoneal injection of isotopic sodium bicarbonate, carboxyl-labeled sodium acetate, and carboxyl-labeled sodium succinate.
2. The cumulative excretion of radioactive carbon amounted in 4 hours to 95 per cent after bicarbonate, 87 per cent after acetate, and 86 per cent after succinate.
3. The rates of change of the specific activity of the  $CO_2$  have been interpreted in terms of the metabolic reactions of the substances injected.
4. The velocity constants for the conversion of acetate and succinate carboxyl carbons to  $CO_2$  and for the excretion of  $CO_2$  have been calculated.

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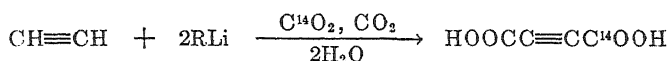
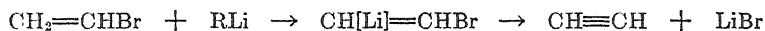
# NOTE ON THE SYNTHESIS OF SUCCINIC ACID LABELED IN THE CARBOXYL POSITION WITH RADIOACTIVE CARBON\*

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(Received for publication, September 4, 1948)

In the course of investigations in intermediary carbohydrate metabolism, it was necessary to employ isotopic succinic acid. This material, labeled with  $C^{14}$  in the carboxyl position, was prepared via acetylenedicarboxylic acid according to the method described by Gilman and Haubein (1). In order to adapt the method to isotopic synthesis modifications were made, as described below, in the carbonation of the Grignard reagent. The reactions involved in the synthesis of the acetylenedicarboxylic acid, as postulated by Gilman and Haubein, are the following:



Succinic acid was then prepared from the acetylenedicarboxylic acid by catalytic hydrogenation.

## EXPERIMENTAL

*Vinyl Bromide and *n*-Butyllithium*—A solution of 3.2 gm. (0.03 mole) of vinyl bromide (prepared in accordance with the directions of Kharasch and coworkers (2)) dissolved in 30 ml. of ether cooled to 0° was added to 0.10 mole of *n*-butyllithium in 200 ml. of ether at 0°. This mixture was stirred for 15 minutes.

*Carbonation of Dilithium Acetylide and Isolation of Acetylenedicarboxylic Acid*—10 ml. of the milky white dilithium acetylide solution were added to  $3.3 \times 10^{-4}$  mole of carbon dioxide which had been generated from 90 mg. of  $BaCO_3$  and 10 mg. of  $BaC^{14}O_3$  (containing approximately 3 per cent  $C^{14}$ ). After cooling the mixture at  $-35^\circ$  for 24 hours, 1.5 gm. of dry ice were added, and, when the latter had all disappeared, 1 gm. of non-isotopic acetylenedicarboxylic acid was introduced as a carrier. Following hydrolysis with 10 ml. of 10 per cent sulfuric acid at 0°, the acid was extracted with eight 10 ml. portions of ether. The ether was removed and the solid residue taken up in 5 ml. of water. Finally, 2 ml. of 20 per cent potassium

\* This work was supported by a contract between Harvard University and the Office of Naval Research.

hydroxide were added; crystallization of monopotassium acetylenedicarboxylate was permitted to continue at 0° for 15 hours. The material was then filtered by suction and washed with acetone. The free acid was obtained by dissolving the salt in 10 ml. of 10 per cent sulfuric acid and extracting with eight 10 ml. portions of ether. Subsequent to drying over sodium sulfate, the ether was removed and the product, after being dried *in vacuo* over sulfuric acid, weighed 685 mg.

*Catalytic Reduction of Acetylenedicarboxylic Acid*—Acetylenedicarboxylic acid (685 mg.) and 250 mg. of 10 per cent platinum-charcoal were added to 15 ml. of water, and the resulting mixture was shaken in an atmosphere of hydrogen at room temperature and atmospheric pressure. 2 moles of hydrogen per mole of acetylenedicarboxylic acid were consumed in about 3 hours. The residue obtained after filtration and evaporation to dryness *in vacuo* was recrystallized from water. The yield of succinic acid in this step was quantitative, and the melting point was 185°. The over-all yield of C<sup>14</sup> in succinic acid from the BaC<sup>14</sup>O<sub>3</sub> added was 11 per cent.

#### SUMMARY

Succinic acid, labeled with C<sup>14</sup> in the carboxyl position, has been prepared from BaC<sup>14</sup>O<sub>3</sub> via acetylenedicarboxylic acid.

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# SEDIMENTATION, DIFFUSION, AND MOLECULAR WEIGHT OF CRYSTALLINE PITUITARY GROWTH HORMONE\*

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(Received for publication, September 7, 1948)

The recent work of Fishman, Wilhelmi, and Russell (1, 2) has made available a crystalline growth hormone isolated from the bovine anterior pituitary gland. It was of considerable interest to determine the sedimentation behavior of this protein in the ultracentrifuge and, at the same time, to obtain the molecular weight by this method. The amorphous preparation of Li, Evans, and Simpson (3) has not been studied in the ultracentrifuge, although estimations of the molecular weight have been obtained by other methods (3-5).

The lyophilized samples of the crystalline growth hormone were prepared as already described (2). The material was electrophoretically homogeneous at pH 4.0 and at pH 10.0. When the hormone was injected daily for 10 days into hypophysectomized male rats weighing 100 gm., it produced, at a daily dose of 20  $\gamma$ , an average increase in body weight of 15.8 gm. and, at a daily dose of 100  $\gamma$ , an increase in body weight of 25.0 gm. The corresponding increments in the width of the proximal epiphyseal cartilage of the tibia were 180  $\mu$  and 207  $\mu$ .

## *Ultracentrifuge Studies*

*Methods*—Our observations were made in the Spinco<sup>1</sup> ultracentrifuge designed by Dr. Edward G. Pickels. Since no description of the design, precision, or controls of this instrument has been given, we are presenting a brief account of its main characteristics. We are greatly indebted to Dr. Pickels for his courtesy and help in preparing this summary.

The drive of the Spinco ultracentrifuge consists of a 110 volt, series-wound, brush type, electrical motor of 1.5 horse power rating, high speed spindle and low speed take-offs to a speed control mechanism, and an electrical tachometer. The spindle speed is  $5\frac{1}{2}$  times that of the electrical motor. The drive is kept at a safe operating temperature by a forced air draft and a system of water cooling for both the motor and the bearings.

\* This investigation was aided by a grant from the United States Public Health Service.

<sup>1</sup> Specialized Instruments Corporation, Belmont, California.

Heating of the rotor is kept to a minimum by operating it in a vacuum chamber at pressures below  $1\ \mu$  and by interposing a non-conducting bushing of bakelite between the rotor and the drive shaft. The complete drive is mounted on shock-absorbing neoprene and the drive shaft itself is of spring steel 0.1 inch in diameter. This permits the rotor to be self-balancing and insures an almost complete absence of rotor vibration, which is desirable for undisturbed sedimentation and accurate photographic measurements. Disturbance from building vibrations is minimized by mounting the light source and the complete optical system rigidly with respect to the vacuum chamber and a surrounding barricade and floating this entire assembly on neoprene shock mounts.

The rotor is of duralumin and has a maximum diameter of 7.25 inches. Being made of a material with high thermal conductivity, the rotor is ideal from the standpoint of minimizing any temperature gradients which might cause convection within the solution under study. The dimensions of the rotor and cell are patterned after those found most suitable for routine work by Svedberg and his associates. The fluid column of 15 mm. maximum length (measured radially) is at a mean distance of 65 mm. from the axis of rotation. The heating of the rotor is normally of the order of  $1^\circ$  per hour. The temperature of a run is taken as the mean of starting and final temperatures measured with a contact thermocouple (copper-constantan) and a commercial meter having a sensitivity of 1 millivolt for full scale deflection. Temperatures are measured to within  $0.2^\circ$ .

The optical system is of the Philpot-Svensson type employing a cylindrical lens and an inclined bar  $1/16$  inch wide. The total length of the system is slightly less than 3 meters. To eliminate parallax, light from the slit source is passed through the rotor by a collimating lens. A condensing lens above the rotor converges the light back into focus to form an image of the slit at the inclined bar. To avoid readjustment of the optical system for different wave-lengths all lenses are color-corrected, except the cylindrical lens, for which the chromatic errors are negligibly small when the system is separately calibrated for the different wave-lengths used. The displacement of a sharply defined boundary from the meniscus can be determined with an accuracy of 0.1 or 0.2 mm. Accurate determinations of the base-line and also the distance of the meniscus from the axis of rotation are made possible by two reference holes in the counterbalance. One of these shows above and the other below the fluid column in the sedimentation photographs.

Sedimentation during acceleration is kept to a minimum by virtue of the rapidity with which the rotor is accelerated. With a full accelerating current of 15 amperes it reaches 60,000 R.P.M. in less than 7 minutes. It can be decelerated to rest in approximately the same length of time. Operating

speeds are kept constant by a speed control device and it provides for the selection of any one of thirty different speeds, ranging from 12,590 to 74,070. In the speed control mechanism, rotary motion obtained from a synchronous motor and reduced by a selected gear ratio is matched through a differential gear and slip clutch against a low speed rotary motion from the drive. Any difference in speed of the two rotary motions causes movement of the differential which activates electrical switches that control the power to the drive. The speed may drift momentarily through a range of the order of  $\pm 0.5$  per cent of the selected speed, but the average speed over

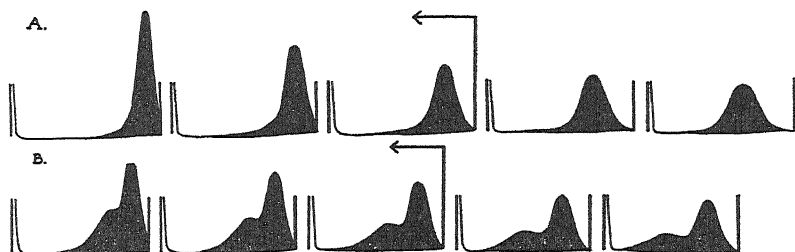


FIG. 1. The upper curves (A) are the diagrams of a 1 per cent solution of the sedimenting growth hormone obtained in glycine buffer of pH 9.49 and at an ionic strength of 0.1 at a speed of 59,780 r.p.m. The first picture on the left was taken 20 minutes after attaining full speed; subsequent photographs were made at 16 minute intervals. The lower curves (B) are the patterns obtained in acetate buffer at pH 4.03 and at an ionic strength of 0.1. The photographs were taken 32 minutes after attaining full speed (59,780 r.p.m.), and at 8 minute intervals thereafter. The patterns show two sedimenting particles where the heavier material apparently is an aggregate of the original hormone. The arrow indicates the direction of sedimentation from the meniscus.

a period of 15 minutes or more is controlled with an accuracy of about 0.1 per cent. An independent check of the speed is made by timing a revolution counter, which is attached directly to a low speed take-off from the drive.

The timings of the photographs and the exposure are controlled automatically by means of adjustable cams operated at reduced speed by a synchronous motor. The accuracy is within about 1 second.

*Results*—Fig. 1 shows the results of two typical runs on the crystalline growth hormone. The upper diagrams illustrate the sedimentation pattern for a run made in glycine buffer. In the alkaline solution at about pH 9.5, the protein sediments with the characteristic boundary of a single molecular species, and no inhomogeneity could be detected. The behavior of the protein in acetate buffer at about pH 4 is quite different (lower curve of Fig. 1). In addition to the component observed in alkaline solution, there are present variable amounts of a more rapidly sedimenting particle. It is

noteworthy that Li (4, 5) has already reported that at this pH the hormone shows viscosity changes indicative of an increase in molecular weight.

Table I summarizes the results obtained. Both components observed in the acetate buffer give variable sedimentation constants. In contrast to these findings, the five independent determinations made in glycine buffer yield values in close agreement with one another. The average value for  $S_{20,w}$  is  $3.60 \pm 0.10 \times 10^{-13}$ . The observations made at different protein concentrations do not show any significant variation in the sedimentation constant.

TABLE I  
*Sedimentation of Crystalline Pituitary Growth Hormone*

Both the glycine and acetate buffers were 0.1 ionic strength. The temperature is the average for the duration of the run. The values of  $S_{20,w}$  incorporate the usual corrections for the density and viscosity of the medium (7). The numbers in parentheses in the first column indicate the two different preparations.

Solvent	pH	Protein concentration	Temperature	$S_t \times 10^{13}$	$S_{20,w} \times 10^{13}$
		per cent	°C.		
Glycine (1)*	9.49	1.0	26.4	4.01	3.62
" (1)	9.35	0.5	26.2	3.67	3.38
" (2)	9.70	1.2	14.9	3.13	3.73
" (2)	9.55	0.6	21.0	3.60	3.70
" (2)	9.67	0.3	19.9	3.38	3.55
0.4 M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (2)	5.81	1.0	24.2	3.09	3.26
Acetate (1)	3.85	1.0	26.1	4.07	3.65 (28%)
				13.1	11.8 (72%)
" (2)*	4.03	1.0	24.5	3.32	3.09 (57%)
				6.85	6.15 (43%)
" (2)†	4.03	1.0	25.1	2.79	2.56 (50%)
				6.79	6.23 (50%)

\* Shown in Fig. 1.

† This was the same as the preceding sample, but it had been allowed to stand overnight at 5°.

One run in 0.4 M ammonium sulfate solution indicated a single component, but it had a somewhat lower sedimentation constant than that found in the glycine buffer. However, another run in this solvent, not given in Table I, showed about 10 per cent of heavier material.

#### *Diffusion Studies*

Diffusion constants were measured in the electrophoresis cell by the procedure described by Longworth (6) from photographs taken by the schlieren scanning method. Results were computed from the formula,

$D = (A^2)/(4\pi tH^2)$ , where  $A$  is the area under the curve in sq. cm.,  $H$  the maximum height of the boundary in cm.,  $t$  the time in seconds, and  $D$  the diffusion constant in sq. cm. per second.

Two separate runs at a protein concentration of 0.7 and 1.1 per cent, respectively, were performed at 1.5° in glycine buffer at pH 9.6 and at an ionic strength of 0.1. Each run was carried out in duplicate, both halves of the cell being used separately. From five to seven photographs were taken at intervals from about 7 to 50 hours. Plots of  $1/H$  against  $t$  gave excellent straight lines passing through the origin, indicating that no boundary disturbance had occurred.

The average value for the first run was  $D_{1.5,G} = 4.01 \times 10^{-7}$ , and for the second run  $4.00 \times 10^{-7}$ . The values for  $D_{20,w}$  were found by correcting for the temperature and viscosity differences in the usual manner (7). We obtained  $D_{20,w} = 7.37$  and  $7.35 \times 10^{-7}$ , giving an average of  $7.36 \times 10^{-7}$  sq. cm. per second.

The diffusion constant found for the crystalline hormone is in good agreement with the  $D_{20,w} = 7.15 \times 10^{-7}$  obtained by Li (4) with the amorphous preparation.

### *Molecular Weight*

This was calculated from the usual sedimentation-velocity formula (7) where  $M = RTs/D(1 - V\rho)$ , with the average values for  $s$  and  $D$  reported above and  $V = 0.76$  as found by Li (4). The molecular weight of the growth hormone is found to be 49,200.

Our result may be compared with the various estimates of the molecular weight obtained by other methods. Li, Evans, and Simpson (3) found 44,250 by osmotic pressure measurements; Li (4) reported 39,300 from diffusion-viscosity measurements, and an average value of 47,300 from amino acid analysis (5). It is apparent that the molecular weight obtained by the sedimentation-diffusion method is in best accord with the results found by analysis, although all of the methods are in fair agreement.

### SUMMARY

1. The crystalline growth hormone obtained from bovine anterior pituitary glands is completely homogeneous in alkaline solutions as observed by its sedimentation behavior in the ultracentrifuge. In acid solutions, about pH 4, the protein tends to produce aggregates of larger size.
2. From the sedimentation constant,  $S_{20,w} = 3.60 \times 10^{-13}$ , and the diffusion constant,  $D_{20,w} = 7.36 \times 10^{-7}$ , the molecular weight of the hormone has been computed to be 49,200.

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# THE HEXOKINASE REACTION IN TISSUE EXTRACTS FROM NORMAL AND DIABETIC RATS\*

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(Received for publication, July 31, 1948)

The recent papers by Cori and his coworkers have stimulated interest in the hexokinase reaction in tissue extracts from normal and pathological animals.

Our interpretation of this work pertinent to the subject of this paper is as follows: (1) Rats injected with alloxan yield muscle extracts which show an initial lag of hexokinase activity of 10 to 15 minutes duration, owing to the presence in muscle from diabetic animals of a labile inhibitory factor from the pituitary gland (1). (2) The initial slow phase in muscle extracts from alloxanized rats is abolished by the prior addition of insulin (2). (3) The addition of Upjohn's adrenal cortical extract (ACE) to muscle extracts from alloxanized rats often markedly intensifies the initial slow phase of the hexokinase reaction. This is presumed to be caused by an intensification of the inhibitory action of the pituitary factor present in diabetic tissue. This added effect of adrenal cortical hormone is always prevented by the prior addition of insulin (2, 3). (4) Muscle extracts from alloxanized animals subject to mild aging (45 to 270 minutes at 0°) are not affected by the addition of ACE. The hexokinase reaction is similar in rate to that obtained with fresh extracts in the presence of insulin. Presumably a labile factor is lost during the aging process; in this respect it resembles a factor present in preparations from the anterior pituitary gland (3). (5) In a series of thirty rats given alloxan, and considered to be diabetic, fifteen yielded muscle extracts which, in the presence of ACE, gave rates of the hexokinase reaction which ranged from 24 to 79 per cent of those obtained with the same extracts in the presence of ACE + insulin. These decreases of hexokinase rate were considered significant (3).

We report in this paper similar experiments on tissue extracts from normal rats and alloxanized rats.

## *Methods*

*Rats*—The white rat was used throughout. In early experiments selection as to strain, source, weight, environment, etc., was not rigid. In later

\* The work reported in this paper was assisted in part by grants from the National Institute of Health, United States Public Health Service, and the Insulin Grant Committee of the Lilly Research Laboratories.

† Fellow of the American Chemical Society.

experiments groups of rats were carefully selected to make these factors as uniform as possible. The animals were fed *ad libitum* on standard rat food pellets.

*Alloxanization*—The rats were injected intraperitoneally with 200 mg. of alloxan per kilo. Weight, food intake, urine volume, glucose excretion per day, and the appearance of ketone bodies in the urine were followed. The blood glucose was determined immediately before an experiment. The alloxanized rats used in the experiments fall in two groups: (1) alloxanized 3 to 20 days (average 7 days) before use; (2) alloxanized 48 hours before use.

*Preparation of Tissue Extracts. Muscle*—The rats were decapitated and the muscles removed from the hind legs as rapidly as possible and collected on ice. In the cold room the muscle was blotted with filter paper, weighed, and minced lightly with scissors on a cold porcelain plate. The mince was ground in a mortar with a small amount of sand for 4 minutes, 3 volumes of water ( $0^{\circ}$ ) being added in small portions during the grinding. The suspension was centrifuged for 3 minutes at 3000 R.P.M. and then strained through cheese-cloth. In separate reaction vessels were added, in order, 0.2 ml. of 0.45 M  $\text{NaHCO}_3$ , 0.2 ml. of Upjohn's adrenal cortical extract (where indicated), 0.2 ml. of an insulin solution containing 30 units per ml. (where indicated), 2.4 ml. of extract, 0.2 ml. of 0.25 M  $\text{MgCl}_2$ . This completed the preparation of the samples at  $0^{\circ}$ .

Every effort was made to cut the preparation time to a minimum and to maintain the preparations at a low temperature. In the great majority of cases the ice-cold extract, prepared as above, was placed in a bath at  $30^{\circ}$  within 15 minutes after the decapitation of the rat; in no case did this time exceed 18 minutes. These precautions were taken to minimize the destruction with time and temperature of any possible labile factors present in the muscle.

*Kidney*—The kidney extracts were prepared in the same way except that 5 volumes of water were used. The kidneys were kept at  $0^{\circ}$  for about 30 minutes before the extract was prepared; *i.e.*, during the time the experiments with muscle were started.

#### *Measurement of Hexokinase Reaction*

*Initiation of Reaction*—The reaction vessel containing the extract prepared as above was placed in a bath at  $30^{\circ}$  and gassed with 95 per cent  $\text{N}_2$  and 5 per cent  $\text{CO}_2$ . 5 minutes later 2 ml. of the following medium were added: 0.016 M ATP,<sup>1</sup> 0.010 M glucose, 0.03 M  $\text{NaHCO}_3$ , and (omitted in some experiments) 0.06 M NaF. The final concentrations in the reaction system were as follows: 0.030 M  $\text{NaHCO}_3$ , 0.010 M  $\text{MgCl}_2$ , 0.024 M NaF,

<sup>1</sup> Adenosine triphosphate.



0.004 M glucose, 0.0064 M ATP; when present, insulin 1.2 units per ml., ACE 0.04 ml. per ml. The vessels were shaken at a moderate rate during the subsequent course of the experiment.

*Glucose Analysis*—Exactly 1 minute after the addition of ATP-glucose medium the first sample was taken for analysis. This is the zero time recorded in all tables. Further samples were taken as indicated in the tables.

The 0.5 ml. samples were added to iced solutions of 2 ml. of 5 per cent  $\text{ZnSO}_4$ ;  $\text{Ba}(\text{OH})_2$  was then added to give a neutral filtrate. Control experiments showed that no organic phosphate appeared in the filtrates. The solutions were diluted to 10 ml. and aliquots analyzed for glucose by the Benedict method.

*Formulation of Data*—The regression coefficient of glucose on time was calculated by conventional statistical methods. This gives the hexokinase activity expressed as the rate of decrease of glucose which is expressed as  $1000 \times$  micromoles of glucose per ml. of extract per minute. The statistical presentation of the data follows closely the methods outlined by Snedecor (4).

The following abbreviations are used in the statistical presentation:  $N$  = number of observations;  $\sigma$  = standard deviation; s.e. = standard error;  $\bar{X}$  = mean;  $\Delta$  = difference; d.f. = degrees of freedom;  $t$  = Fisher's ratio for determining the level of significance;  $F$  = Snedecor's variance ratio for determining the level of significance.

*Reagents*—Two adenosine triphosphate preparations were used: one prepared from rabbit muscle as the Ba salt according to Kerr (5); the other a commercial preparation from the Schwarz Laboratories, New York.

The insulin used was a Lilly amorphous preparation. Adrenocortical extract was obtained from The Upjohn Company.<sup>2</sup> The extract was concentrated to about half the original volume by evaporation *in vacuo* in order to remove the alcohol which is present as a preservative, and rediluted. Glass-distilled water was used in all experiments.

#### *Hexokinase Reaction in Muscle Extracts from Normal and Alloxanized Rats*

In the first series, a group of nine rats which had received alloxan 3 to 20 days prior to the experiment was used. Six rats from the same batch were used as controls. The data are shown in Table I. All samples contained ACE. In addition others contained insulin, or were "aged" at 30° for 30 minutes.

*Effect of Insulin*—Within groups, there is no effect, as indicated by the non-significant values of  $F$ , in either normal or alloxanized rats. Further-

<sup>2</sup> We are indebted to Eli Lilly and Company and The Upjohn Company for their generous donation of these preparations.

TABLE I  
*Hexokinase Reaction in Extract of Muscle from Normal and Alloxanized Rats*

Bicarbonate, ATP, glucose, NaF medium; gas phase N<sub>2</sub> + 5 per cent CO<sub>2</sub>; temperature 30°; Upjohn adrenal cortical extract, 0.04 ml. per ml., in all; Lilly amorphous insulin, 1.2 units per ml., where indicated. Extracts aged for 30 minutes at 30° where indicated.

The values given are the regression coefficients plus or minus the standard error (s.e.) of the regression calculated from five determinations of glucose at 0, 5, 10, 20, and 40 minutes.

Rat No.	Normal rats				Alloxanized rats			
	Rates, 1000 X micromoles per ml. extract per min.				Rates, 1000 X micromoles ml. per extract per min.			
	ACE	ACE + insulin	ACE, aged	Subtotal	ACE	ACE + insulin	ACE, aged	Subtotal
1	69 ± 3	97 ± 16	123 ± 10		96 ± 15	71 ± 12	89 ± 18	
2	116 ± 15	100 ± 7	94 ± 14		83 ± 2	70 ± 11	78 ± 20	
3	71 ± 6	68 ± 8	98 ± 11		7 ± 13	34 ± 3	49 ± 5	
4	69 ± 10	97 ± 2	107 ± 4		47 ± 7	39 ± 8	70 ± 8	
5	129 ± 3	132 ± 12	30 ± 16		2 ± 4	16 ± 12	29 ± 13	
6	62 ± 8	53 ± 3	51 ± 31		63 ± 2	54 ± 10	36 ± 16	
7					184	1.7	56 ± 17	
8					140	1.7	31 ± 13	
9					987	0.7	78 ± 11	
					1545	0.3	188 ± 23	
Mean.....	86	91	84	83	68	62	72	67
s.e. of mean...	17	17	17	10	14	14	14	8

Variances				Fiducial intervals ( $P = 0.05$ )
	D.f.	$\sigma^2$	F	
Normals				
Between groups	2	78		
Within "	15	953	0.08*	Between two extracts from same rat (d.f. = 135)
Alloxanized				
Between groups	2	833		
Within "	24	2303	0.36*	Between normal and alloxanized means (d.f. = 39)
Normals + alloxanized				
Between groups	5	1178		
Within "	39	1779	0.66*	
Pooled variance of regression	135	(13) <sup>2</sup>		
error				
Pooled s.e. of mean	39	$\sqrt{1779/N}$		

\*  $P > 0.05$ .

TABLE II  
*Hexokinase Reaction in Extracts of Muscle from Normal Rats, and from Rats 48 Hours after Alloxanization*

Bicarbonate, ATP, glucose, NaF medium; gas phase N<sub>2</sub> + 5 per cent CO<sub>2</sub>; temperature 30°; Upjohn adrenal cortical extract 0.04 ml. per ml. where indicated; Lilly amorphous insulin 1.2 units per ml. where indicated. The values given are the regression coefficients  $\pm$  s.e. of the regression calculated from four determinations of glucose at 0, 5, 25, 30 minutes.

Rat No.	Normal rats				Alloxanized rats					
	Rates, 1000 $\times$ micromoles per ml. extract per min.				Blood sugar mg. per 100 gm. per 24 hrs.	Glycosuria	Rates, 1000 $\times$ micromoles per ml. extract per min.			
	Plain	ACE	ACE + insulin	Subtotal			Plain	ACE	ACE + insulin	Subtotal
1	150 $\pm$ 9	141 $\pm$ 4	112 $\pm$ 2		119	0	126 $\pm$ 7	122 $\pm$ 8	144 $\pm$ 8	
2	174 $\pm$ 5	176 $\pm$ 2	154 $\pm$ 6		628	0.9	118 $\pm$ 28	115 $\pm$ 20	95 $\pm$ 10	
3	195 $\pm$ 0	169 $\pm$ 11	137 $\pm$ 6		470	0.5	154 $\pm$ 10	145 $\pm$ 6	154 $\pm$ 15	
4	203 $\pm$ 6	208 $\pm$ 3	175 $\pm$ 6		306	1.8	188 $\pm$ 9	163 $\pm$ 2	156 $\pm$ 3	
5	197 $\pm$ 4	146 $\pm$ 23	145 $\pm$ 6		162	0.1	154 $\pm$ 11	166 $\pm$ 14	153 $\pm$ 11	
6	180 $\pm$ 6	166 $\pm$ 8	135 $\pm$ 11		438	2.0	159 $\pm$ 6	166 $\pm$ 9	157 $\pm$ 6	
7					151	0.1	105 $\pm$ 10	116 $\pm$ 6	103 $\pm$ 4	
8					388	1.6	128 $\pm$ 3	130 $\pm$ 4	137 $\pm$ 21	
9					198	0	215 $\pm$ 10	202 $\pm$ 16	187 $\pm$ 13	
Mean . . . . .	183	178	144	164			149	147	143	147
S.E. of mean . . . . .	12	12	12	6.8			10	10	10	4.3

Variances						Differences					
Normal			Alloxanized		Both		D.f.	$\Delta$	$\sigma_{\Delta}$	$t$	
D.f.	$\sigma^2$	D.f.	$\sigma^2$	D.f.	$\sigma^2$						
Between groups	2	2464	2	105	5	1763	Normal (plain and ACE) (ACE + insulin) Normal, alloxanized Plain ACE " + insulin All Plain and ACE " " " (all)	36	15	2.40*	
Within	15	527	24	969	39	834					
$F_{\text{obs.}}$		5.27*		0.11†	90	2.11† (11) <sup>2</sup>		34	15	2.26†	
Pooled variance of regression error								31	15	2.07†	
$\sigma \bar{x}_1 - \bar{x}_2$					39	$\sqrt{834(N_1 + N_2)/N_1 \times N_2}$		13	15	0.07†	
							43	17	11	1.55†	
							28	32	11	2.91†	
							37	33	10	3.30†	

\*  $P = 0.01$  to  $0.05$ .†  $P > 0.05$ .‡  $P < 0.01$ .

more, within rats there is no ACE + insulin extract which exceeds its ACE control by the fiducial interval of 37.

*Effect of Aging*—The possibility of the existence in the extracts of a labile inhibitory factor which would be destroyed by mild aging at 30° for 30 minutes was tested. There is no indication from the group means that this aging process has any effect in normal or alloxanized rats. Within rats, a single "ACE, aged" extract in the alloxanized group gave a hexokinase rate exceeding its ACE control by the fiducial interval of 37. In the normal group, two "ACE, aged" extracts were higher, and only one lower by 37 than their respective controls. No significance is attached to these findings.

*Effect of Alloxanization*—There is no indication from the group means  $\pm$  S.E., as well as the  $F$  value of the combined groups, that alloxanization has any effect on the rate of the hexokinase reaction. The variances of the normal and alloxanized groups are significantly different ( $F = 2.42$ ;  $P < 0.05$ ,  $> 0.01$ ). This significantly greater variability of the alloxanized group remains unexplained; it was not encountered again in our experiments. However,  $\chi$  square tests show that all subgroups are homogeneous.

*Blood Sugar, Glycosuria, and Hexokinase Rate*—The difficulties of determining the diabetic state of alloxanized rats is well known. However, with high blood sugar as a rough index, significant glucose excretion, or both, we conclude that eight of these nine rats (Rat 2 being excepted) were diabetic. There is no correlation between the extent of departure from normality as indicated by these criteria with the rate of the hexokinase reaction, the effect of insulin, or aging.

#### *Hexokinase Reaction in Muscle Extract from Rats 48 Hours after Alloxanization*

Rats were carefully selected for similarity in weight, strain, environment, feedings, etc., and used 48 hours after alloxanization. The data are shown in Table II. Of the alloxanized group, only Rat 1 showed no hyperglycemia or glycosuria.

#### *Effect of ACE, or ACE Plus Insulin*

*Normal Rats*—The  $F$  value indicates a significant difference among the groups due to the fact that the ACE + insulin group is different from the plain and ACE groups. This is an unusual finding and raises a difficulty in the subsequent evaluation of the data.

*Alloxanized Rats*—The means show no effect of ACE or ACE + insulin. In no case within rats does a single extract with ACE or ACE + insulin show a significant difference from its plain control.

*Effect of Alloxanization*—The table of differences shows that taken separately the plain and ACE groups of the normal and the alloxanized rats give

high, but not significant *t* values. When these two groups are pooled, there is a significant difference between the normal and the alloxanized series. However, the normal ACE + insulin is not significantly different from any combination of alloxanized groups. These ambiguous results make interpretation of the data difficult. Conservatively, it can be concluded that the data show a tendency for alloxan to diminish the hexokinase reaction, but final decision must depend on further evidence.

TABLE III

*Hexokinase Reaction in Extracts of Muscle from Normal Rats and from Rats 48 Hours after Alloxanization; Adrenal Cortical Extract and Insulin Omitted*

Bicarbonate, ATP, glucose, NaF medium; gas phase N<sub>2</sub> + 5 per cent CO<sub>2</sub>; temperature 30°. The values given are the rates of decrease of glucose calculated from two analyses at 0 and 30 minutes.

Rat No.	Normal rats	Alloxanized rats		
	Rates	Blood sugar	Glycosuria	Rates
	1000 × micromoles per ml. extract per min.	mg. per 100 ml.	gm. per 24 hrs.	1000 × micromoles per ml. extract per min.
1	133	135	0.1	150
2	70	913	0.0	130
3	193	554	0.5	130
4	201	132	0.1	210
5	120	471	0.9	140
6	113	202	0.7	180
7	133	108	0.1	80
8	150	552	0.0	100
9	157	510	0.1	120
10	97	481	0.8	0
11	163	470	0.1	134
12	107			
Mean.....	125			125
Variance.....	3044			2953
S.E. of mean.....	16			17

A third series of rats (Table III) selected for similarity in weight, environment, and strain was tested 48 hours after alloxanization. A similar group was used for controls. Eight out of eleven alloxanized rats had blood sugars higher than 200 mg. per 100 ml. Glycosuria was low in all cases. Since we had failed to show any effect of ACE or ACE + insulin in previous series, the experiment was limited to a comparison of the rates without these additions. The data are strikingly similar in means and variances, showing that alloxanization had no effect on the hexokinase reaction in this series.

*Hexokinase Reaction of Muscle Extracts from Rats 48 Hours after  
Alloxanization in the Presence of ACE; Sodium Fluoride  
Absent in Reaction Medium*

Of a group of twenty-four selected rats, twelve were given alloxan 48 hours before the experiment. All of the alloxanized rats showed glycosuria or hyperglycemia. The assay of hexokinase in the muscle extracts was

TABLE IV

*Hexokinase Reaction in Extracts of Muscle from Normal Rats and from Rats 48 Hours  
after Alloxanization; No Sodium Fluoride in Medium, Adrenal  
Cortical Extract Present in All Samples*

Bicarbonate, ATP, glucose medium; gas phase  $N_2 + 5$  per cent  $CO_2$ ; temperature  $30^\circ$ . The values given are the regression coefficients  $\pm$  s.e. of the regression calculated from four determinations of glucose at 0, 5, 25, and 30 minutes.

Rat No.	Normal rats	Alloxanized rats		
	Rates	Blood sugar	Glycosuria	Rates
	<i>1000 <math>\times</math> micromoles per ml. extract per min.</i>	<i>mg. per 100 ml.</i>	<i>gm. per 24 hrs.</i>	<i>1000 <math>\times</math> micromoles per ml. extract per min.</i>
1	135 $\pm$ 10	147	0.1	172 $\pm$ 6
2	106 $\pm$ 14	598	0.0	117 $\pm$ 5
3	131 $\pm$ 5	396	1.0	130 $\pm$ 13
4	97 $\pm$ 8	368	0.6	165 $\pm$ 7
5	122 $\pm$ 21	162	0.3	163 $\pm$ 10
6	152 $\pm$ 8	487	1.6	123 $\pm$ 8
7	126 $\pm$ 7	358	2.4	132 $\pm$ 9
8	148 $\pm$ 0	152	0.1	158 $\pm$ 8
9	114 $\pm$ 8		1.9	98 $\pm$ 14
10	157 $\pm$ 12	340	1.3	186 $\pm$ 17
11	208 $\pm$ 3	382	0.4	164 $\pm$ 9
12	142 $\pm$ 11	109	0.1	132 $\pm$ 19
Mean . . . . .	136			145
Variance . . . . .	841			700
Pooled variance of regression error				(11) <sup>2</sup>

made in the absence of NaF; otherwise, the medium was the same as that hitherto used.

The data are shown in Table IV. There is no indication, on comparison with the control group, that the diabetic state brought about by alloxan has influenced the hexokinase rate. Comparison with the previous series carried out in the presence of NaF shows that omission of this reagent has no effect in rat muscle extracts on the assay value of the hexokinase reaction.



*Hexokinase Reaction in Kidney Extracts from Normal and Alloxanized Rats*

Extracts similar to those prepared from muscle from the kidneys of the rats included in Table I were used to determine the hexokinase reaction in the presence of ACE and ACE + insulin. The data are shown in Table V.

TABLE V

*Hexokinase Reaction in Extracts of Kidney from Normal and Alloxanized Rats; Adrenal Cortical Extract Present in All Samples*

Bicarbonate, ATP, glucose, NaF medium; gas phase  $N_2 + 5$  per cent  $CO_2$ ; temperature  $30^\circ$ . The values given are the regression coefficient  $\pm$  s.e. of the regression calculated from five determinations of glucose at 0, 5, 10, 20, and 40 minutes. The rats correspond to those included in Table I.

The rates are expressed in  $1000 \times$  micromoles per ml. of extract per minute.

Rat No.	Normal rats		Alloxanized rats	
	ACE	ACE + insulin	ACE	ACE + insulin
1	82 $\pm$ 18	55 $\pm$ 19	76 $\pm$ 12	67 $\pm$ 12
3	68 $\pm$ 7	45 $\pm$ 4	50 $\pm$ 8	37 $\pm$ 13
4	51 $\pm$ 16	82 $\pm$ 16	55 $\pm$ 24	31 $\pm$ 9
5	52 $\pm$ 5	42 $\pm$ 4	100 $\pm$ 10	82 $\pm$ 20
6	88 $\pm$ 15	61 $\pm$ 12	36 $\pm$ 8	39 $\pm$ 14
7			54 $\pm$ 9	51 $\pm$ 26
Mean . . . . .	68	57	62	51
s.e. of mean. . . . .	8	8	8	8

## Variance; all groups

	D.f.	$\sigma^2$
Between groups . . . . .	3	286
Within groups . . . . .	16	418
$F_{obs.}$ . . . . .		0.69*
Pooled variance of regression error . . . . .	66	(13) <sup>2</sup>
Fiducial interval between two extracts from same rat ( $P = 0.05$ ) = 37		

\*  $P > 0.05$ .

It is obvious that the groups are similar with respect to means and variances and that neither alloxanization nor insulin has any effect upon the hexokinase reaction. Furthermore, no single extract with ACE + insulin exceeds its control with ACE alone by the fiducial interval calculated from the pooled regression error.

TABLE VI  
*Comparison of Initial and Final Rates of Hexokinase Reaction in Extracts of Muscle from Normal and Alloxanized Rats*

The rat numbers correspond to those in Table I. The values recorded are the regression coefficients  $\pm$  s.e. of the regression calculated from the glucose values at 0, 5, 10, and 20 minutes, and at 0, 5, 10, 20, and 40 minutes. The rates are expressed in  $1000 \times$  micromoles per ml. of extract per minute.

Rat No.	Normal rats				Alloxanized rats			
	ACE		ACE + insulin		ACE		ACE + insulin	
	0-20 min.	0-40 min.	0-20 min.	0-40 min.	0-20 min.	0-40 min.	0-20 min.	0-40 min.
1	67 ± 24	69 ± 3	106 ± 21	97 ± 16	82 ± 36	96 ± 15	45 ± 24	71 ± 12
2	125 ± 41	116 ± 15	115 ± 11	100 ± 7	77 ± 0	83 ± 2	91 ± 24	70 ± 11
3	56 ± 10	71 ± 6	82 ± 17	68 ± 8	49 ± 11	7 ± 13	33 ± 10	34 ± 3
4	75 ± 24	69 ± 10	93 ± 19	97 ± 2	55 ± 14	47 ± 7	30 ± 20	39 ± 8
5	125 ± 7	129 ± 3	153 ± 26	132 ± 12	-11 ± 0	2 ± 4	33 ± 28	16 ± 12
6	38 ± 7	62 ± 8	57 ± 7	53 ± 3	66 ± 0	63 ± 2	53 ± 26	54 ± 10
7					42 ± 24	59 ± 13	57 ± 44	56 ± 17
8					103 ± 7	92 ± 5	68 ± 27	51 ± 11
9					189 ± 22	162 ± 12	179 ± 25	159 ± 10
Mean . . . . .	81	86	101	91	72	68	66	62
S.E. of mean . . . .	18	17	18	17	15	14	15	14
Times of sampling, min.	D.f.		Pooled error of estimate about regression line					
			Micromoles glucose		Per cent of mean glucose during period of observation			
0, 5, 10, 20	60		0.33		3.7			
0, 5, 10, 20, 40	90		0.41		5.0			
Fiducial interval of regression coefficient ( $P = 0.5$ ) = 52								
Pooled S.E. of mean. 0-20 min. $\sqrt{2011/N}$								
0-40 " $\sqrt{1779/N}$								
Pooled error of regression coefficient, micromoles glucose per ml. extract per min. $\times 1000$								
22								
13								

*Initial Lag in Hexokinase Reaction*

The data from the experiments in Table I make it possible to determine whether or not the addition of ACE to a muscle extract from alloxanized rats produces an initial slow phase owing to the intensification of an inhibitory factor. It is also possible to test whether the further addition of insulin eliminates such action of a hypothetical inhibitory factor. Any such lag would appear upon comparison of the rates in the early and late periods of the experiment. From the data in Table VI the following conclusions are drawn. (1) The pooled error of estimate about the regression line averages 3.7 to 5.0 per cent of the mean glucose level during the period of observation. (2) Within groups there is no indication that the means of the rates of either the ACE or the ACE + insulin groups are different during the 0 to 20 minute and the 0 to 40 minute periods. (3) Within rats, there is no case, either with ACE or ACE + insulin, in which the initial rate is less by the fiducial interval than the over-all rate. In other words, the data reveal no evidence for any initial lag period in any category. Examination of other series not included in Table VI also shows no evidence of any initial lag period in extracts with or without ACE, ACE + insulin, or insulin.

The authors emphasize that comparison of experiments with tissue extracts in different laboratories is difficult. Unrecognized variations of methods of preparation of the extract and subsequent assay may be important causes of discrepant results. Differences of rat strains, variation of resistance to alloxan, etc., must also be considered. It is possible that the degree of diabetes induced in this series was insufficient to alter the hexokinase reaction. Nevertheless, it is difficult to understand, if depression of the hexokinase reaction occurs in diabetic rats, why muscle or kidney extracts from forty-one alloxanized rats failed to show, either by comparison of groups of rats or by intercomparison of extracts from the same rat, any statistical evidence of such alteration.

## SUMMARY

1. The hexokinase reaction was measured anaerobically by the rate of decrease of glucose at 30° in a system containing tissue extract, bicarbonate, adenosine triphosphate, glucose, magnesium, and sodium fluoride.

2. Extracts of muscle from normal rats and from rats 3 to 20 days after alloxanization were tested. No significant effect of adrenal cortical extract or adrenal cortical extract + insulin was observed in either normal or alloxanized rats. The diabetic state induced by alloxan did not alter the rate of the hexokinase reaction when compared to that of the control groups of rats.

3. Extracts of muscle from rats 48 hours after alloxan showed no effect

of adrenal cortical extract with or without insulin. The hexokinase activity of these diabetic rats was not significantly different from that of the normal controls. The tendency toward lower rates observed in one series of alloxanized rats was not observed in three other series.

4. The hexokinase reaction in muscle extracts in the presence or absence of sodium fluoride was essentially the same. No difference between the hexokinase reaction in extracts from normal and alloxanized rats could be demonstrated by the omission of fluoride.

5. No initial slow phase of the hexokinase reaction was observed in extracts of muscle from normal rats, alloxanized rats, or in extracts of kidney from normal or alloxanized rats. In addition, muscle extracts from normal or alloxanized rats when aged for 30 minutes at 30° were not different from controls when tested for hexokinase activity in the presence of adrenal cortical extract. By these criteria, the data fail to demonstrate the presence in normal or diabetic tissues of a labile inhibitory factor which is intensified in action by adrenal cortical extract.

6. The summation of the data presented in this paper fails to show any alteration of the hexokinase reaction in muscle or kidney extracts from diabetic rats when compared to normal controls.

We wish to express our grateful appreciation to Mrs. Phyllis Stapley Tuddenham for her painstaking care in the performance of much of the analytical work reported in this paper.

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# THE METABOLISM OF CURIUM IN THE RAT\*

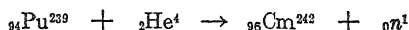
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## PLATES 1 AND 2

(Received for publication, August 2, 1948)

The heaviest of the known elements is curium, which was recently discovered by Seaborg and his associates (1). This new element can be produced by the  $\alpha$ -particle transmutation of plutonium by the following reaction.



This isotope of curium is radioactive and decays by the emission of an  $\alpha$ -particle to form  $\text{Pu}^{235}$  which, in turn, is also radioactive.  $\text{Cm}^{242}$  has a half life of 150 days and its radioactive daughter,  $\text{Pu}^{235}$ , has a half life of 50 years. This isotope of plutonium decays by the emission of an  $\alpha$ -particle to form  $\text{U}^{231}$  which has a half life of 233,000 years.

Shortly after the organization of the Atomic Energy Project, it became apparent that formidable problems would be presented as the result of the release of nuclear energy. One of the most urgent of these was the hazard presented by the production of large quantities of the radioelements created by the fission of uranium and the coincidental formation of neptunium and plutonium. In an attempt to evaluate the potential danger presented by these radioelements from the chain-reacting pile, a large series of metabolic studies with experimental animals was undertaken in a number of laboratories working upon the atomic energy program. These studies, which have been briefly summarized elsewhere (2), included a series of investigations on the metabolism in the rat of the more important members of the fission products in the carrier-free state, as well as most of the heaviest elements at the end of the periodic table. These studies made it possible to predict on a semiquantitative basis the potential hazards that this large number of radioactive elements might present should they gain entry into the body.

Recently a description of the metabolism of americium has been reported in considerable detail (3). This element immediately precedes curium in

\* This document is based on work performed under contract No. W-7405-eng-48-A-I of the University of California under the United States Atomic Energy Commission.

the periodic table and is also radioactive. Americium and curium are not available in sufficient amounts to make them extensive hazards to those working in the field of nuclear energy as compared to the potential dangers presented by the relatively enormous quantities of plutonium and fission products that are produced. Americium and curium resemble plutonium and certain members of the fission products in their chemical and physical properties. An investigation into their metabolic properties was desirable for two reasons, first to evaluate their potential health hazard and, second, to compare their metabolic characteristics with those of plutonium and its chemical analogues.

The tracer studies of americium have shown that this element is accumulated principally in the liver and the skeleton following parenteral administration. The concentration in the liver initially accounts for approximately 55 per cent of the americium which is absorbed from the site of injection. The skeleton accumulates about 25 per cent and the remaining 20 per cent is distributed throughout the other tissues and excreta.

A large fraction of the americium deposited in the liver is lost from that organ rather rapidly and at an approximately exponential rate with a half period of 10 days. The elimination of this element from the liver presumably takes place by way of the bile, since the amount of americium lost from the liver can be accounted for by the quantity that appears in the feces. It seems probable that very little, if any, of the americium deposited in the liver is reabsorbed, since the amount of this element in the skeleton does not change during the period that the liver content is decreasing. The fraction of americium taken up by the skeleton is held in that organ without perceptible loss for time intervals extending to nearly 1 year. The distribution of americium in the bone has been investigated by the radioautographic technique. Apparently americium is deposited primarily in the region of the periosteum and endosteum and about the small blood vessels of the cortical bone. Very little appears to be laid down in the mineralized structure of the skeleton. Other tissues of the body do not show any striking degree of localization or retention of this element. Another point of interest is that americium is not absorbed from the digestive tract to any significant degree.

Another group of elements has been observed to possess almost identical metabolic properties to those of americium. This group of elements, which arise from the fission of uranium and plutonium, includes the rare earths, lanthanum, cerium, praseodymium, and element 61 (2). It is presumed likely that other members of the lanthanide series of the rare earth elements, such as neodymium, samarium, europium, gadolinium, etc., have similar metabolic characteristics. This presumption is based upon the fact that all of these elements are very much alike with respect to most of their chemical properties. The similarity in the metabolic properties of

americium to the lanthanide rare earths is duplicated to a considerable degree by its chemical properties. The principal valence state of americium is  $+3$ , which is also the principal valence state for the rare earths, and it forms insoluble hydroxides, phosphates, carbonates, and fluorides, as do the rare earths.

The very close similarity in chemical properties of the lanthanum group of rare earths to one another is due to the fact that this series of fifteen elements, starting with an atomic number of 57 and ending with 71, has the outer valence electrons arranged essentially the same. The additional electron for each successive element goes into the inner  $4f$  electron shell, which is incomplete. The very minor differences of most chemical and physical properties of the lanthanide series are the result of this phenomenon. Seaborg and his associates have pointed out that a somewhat analogous situation presumably exists in the elements at the far end of the periodic table, starting with element 89, which is actinium (1). The picture is less clear cut for the first few elements of the actinide series, since thorium and protoactinium show considerable similarity in chemical properties to their corresponding lighter homologues, hafnium and tantalum, respectively, in the classical form of the periodic table. The resemblance in chemical properties of uranium to its lighter homologue, tungsten, is slight in most respects. The next two members of the actinide series, neptunium and plutonium, are totally unlike their respective lighter homologues, rhenium and osmium, in the conventional periodic table. With uranium there appears a  $+3$  valence state. While not normally very stable, such a valence state does exist, and  $+3$  uranium possesses chemical properties akin of those of the rare earths. The stability of the  $+3$  state increases, going on to neptunium and plutonium. At the same time the higher oxidation states of uranium, neptunium, and plutonium become increasingly difficult to attain. The theory proposed by Seaborg predicted that americium and curium should demonstrate very stable  $+3$  valence states. This concept has been amply borne out by the study of the chemical properties of these two elements, for the only valence observed in aqueous solution to date, with americium and curium, has been the  $+3$  state. It is predicted that elements beyond curium will continue to exhibit chemical properties very similar to one another and to the lanthanide series of rare earths until element 103 is reached. At this point a sudden transition is predicted with the result that element 104 would be expected to resemble hafnium. This concept of the actinide group is based on the premise that this series of elements continues until all of the vacancies of the  $5f$  electron shell have been filled. An analogous situation with the lanthanide group exists, since this series continues until the incomplete  $4f$  electron shell becomes filled, at which point the last rare earth, lutecium, is followed by hafnium.

In view of this interesting theory, which certainly has many observed

facts to substantiate its validity, a series of animal tracer studies has been undertaken with curium, which is element 96, in order to compare its metabolic properties with those of americium, which it so closely resembles in almost all of its chemical and physical properties. We are indebted to Professor Seaborg and his colleagues for making available to us the curium employed in the tracer studies which are described below.

### *Method*

Adult white rats weighing from 200 to 250 gm. were employed as the experimental animals. Fifteen rats were each given, by intramuscular injection, 1 microcurie of  $\text{CmCl}_3$  in a solution of isotonic saline at pH 5. Three animals were given the same quantity of curium by stomach tube. Two rats received, by intramuscular injection, 10 microcuries each. These two animals were given a larger dose in order to have a sufficient quantity of curium deposited in the skeleton to make it possible to secure satisfactory radioautographs. The fifteen rats which received 1 microcurie by intramuscular injection and the three which were given the curium by stomach tube were divided into groups of three and each group was placed in a metabolism cage which permitted the separate recovery of the urine and the feces at daily intervals. The fifteen animals in the intramuscular series were sacrificed in groups of three at 1, 4, 16, 64, and 256 day intervals. The three animals given curium by stomach tube were sacrificed at 4 days and the two animals for the radioautographic studies were sacrificed at 8 days. The animals were sacrificed by the use of chloroform. The tissues and organs removed for separate assay of their curium content included heart, lungs, liver, kidneys, spleen, digestive tract, muscle, skin, testes, brain, blood, and bone from the uninjected hind leg. The injected leg was removed and assayed separately so as to determine the fraction of administered curium which had not been absorbed from the site of administration. The individual tissues, injected leg, urine, and feces were dried at  $100^\circ$  for 24 hours and ashed at  $500^\circ$  for 48 hours. The skinned carcass, which consisted primarily of the skeleton, muscle, fat, and blood, was dried and ashed as a unit. The skeleton of the carcass was separated from the soft tissue and ashed by sifting the material through a fine screen. The curium was separated from the tissue and excreta ash by coprecipitation with lanthanum fluoride and its radioactivity determined. The procedures employed were identical to the methods of assay used for the experiments with americium (3).

The femurs from the two animals given curium by intramuscular injection for radioautographic studies were sectioned by a histological technique which has been described elsewhere (4). The technique employed made it possible to cut undecalcified sections from 4 to 8  $\mu$  in thickness. These



sections were then placed in contact with photographic film in order to obtain the radioautographic pattern of distribution of curium in the tissue.

### Results

The average values for the distribution of curium in the various tissues and excreta at the 1, 4, 16, 64, and 256 day intervals are shown in Table I. These data give the actual measured values, including the fractions re-

TABLE I  
*Deposition of Curium in Tissues of Rat after Intramuscular Administration into Left Leg*

The values given are in per cent of the dose administered and are averages for three rats at each time interval.

Tissue	1 day		4 days		16 days		64 days		256 days	
	per cent per organ	per cent per gm.	per cent per organ	per cent per gm.	per cent per organ	per cent per gm.	per cent per organ	per cent per gm.	per cent per organ	per cent per gm.
Heart	0.11	0.13	0.09	0.01	0.06	0.09	0.02	0.03		
Lungs	0.25	0.19	0.19	0.13	0.13	0.11	0.11	0.07	0.08	0.02
Spleen	0.08	0.16	0.10	0.17	0.10	0.24	0.09	0.20	0.09	0.15
Blood	0.16	0.01	<0.001		0.01	<0.001	0.01	<0.001	0.01	<0.001
Liver	51.4	6.70	38.1	5.70	18.0	2.83	2.14	0.23	0.95	0.10
Kidney	2.44	1.51	1.47	0.90	1.00	0.69	0.69	0.36	0.41	0.19
Gastrointestinal tract	2.77	0.19	5.48	0.35	1.39	0.06	0.20	0.01	0.07	0.002
Bone	21.6	1.18	27.9	1.40	25.6	1.54	21.1	1.07	19.0	1.17
Muscle	1.86	0.02	1.20	0.02	1.63	0.02	0.90	0.01	0.41	0.003
Left leg*	16.0		15.0		10.8		5.91		4.80	
Skin	1.63	0.07	1.34	0.06	0.94	0.03	0.31	0.01	0.08	0.003
Urine	1.47		1.22		5.10		1.34		5.23	
Feces	2.46		17.7		32.1		50.6		60.2	
Recovery of injected dose, %	102.2		109.8		96.8		83.4		91.4	

\* The curium remaining in the left leg represents the unabsorbed fraction of the injected dose.

maining unabsorbed in the hind leg, which was the site of injection. The recovery value for each group indicates the fraction of the administered curium which could be accounted for by the assay procedures employed. Table II presents the same data which have been corrected for the fraction of curium remaining unabsorbed in the injected leg and the deviation of the recovery value from 100 per cent. In Table II there has been added to the values for the skeleton twice the amount of curium present in the bone

of the uninjected leg. This additional correction was made necessary because the injected leg contained a considerable amount of unabsorbed curium and was therefore assayed separately. The opposite hind leg was removed from the carcass for a separate assay which could be compared with the activity found in the remainder of the skeleton.

The high degree of deposition in the liver and skeleton is the most striking finding noted in this series of studies. An examination of Text-

TABLE II

*Deposition of Curium in Tissues of Rat Corrected for Recovery and for Unabsorbed Balance at Injection Site*

The values are averages for three rats at each time interval.

Tissue	1 day		4 days		16 days		64 days		256 days	
	per cent per organ	per cent per gm.	per cent per organ	per cent per gm.	per cent per organ	per cent per gm.	per cent per organ	per cent per gm.	per cent per organ	per cent per gm.
Heart	0.13	0.15	0.09	0.11	0.07	0.10	0.03	0.04		
Lungs	0.29	0.22	0.20	0.14	0.15	0.13	0.14	0.09	0.09	0.02
Spleen	0.09	0.19	0.11	0.18	0.12	0.28	0.12	0.26	0.10	0.17
Blood*	0.19	0.01	<0.001		0.01	<0.001	0.01	<0.001	0.03	<0.001
Liver	59.6	7.77	40.2	6.01	20.9	3.29	2.76	0.30	1.10	0.12
Kidney	2.83	1.75	1.55	0.95	1.16	0.80	0.89	0.46	0.47	0.22
Gastrointestinal tract	3.21	0.22	5.78	0.37	1.61	0.07	0.26	0.01	0.08	0.002
Bone†	25.1	1.37	29.4	1.48	29.7	1.79	27.2	1.38	21.9	1.35
Muscle‡	2.16	0.02	1.27	0.02	1.89	0.02	1.16	0.01	0.47	0.003
Skin	1.89	0.08	1.41	0.06	1.09	0.03	0.40	0.01	0.09	0.003
Urine	1.71		1.29		5.92		1.73		6.04	
Feces	2.85		18.7		37.3		65.3		69.6	

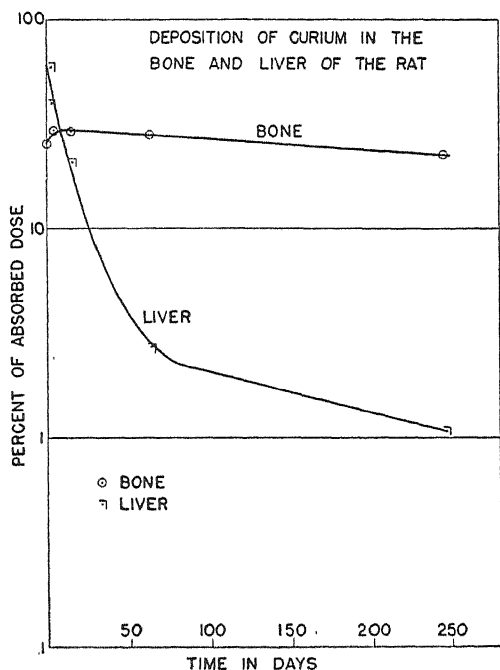
\* The blood was calculated to be 7 per cent of the total body weight.

† The entire skeleton was measured after separation from the balance with a fine mesh screen.

‡ The muscle was calculated to be 45 per cent of the total body weight.

fig. 1 shows that most of the curium accumulated by the liver disappears from that organ quite rapidly. However, the fraction of curium deposited in the skeleton appears to be fixed to a remarkable degree. The bone retention curve, shown in Text-fig. 1, suggests that more than 500 days would be required for the rat to eliminate one-half of the curium deposited in the skeleton from the 4th day after injection. The variations in curium content of the skeleton from the 1 to 256 day intervals, after the appropriate corrections were made, are felt to be within the range of the experimental error. The kidney and spleen were the only other tissues observed that appeared to possess any significant tendency for the preferential accumula-

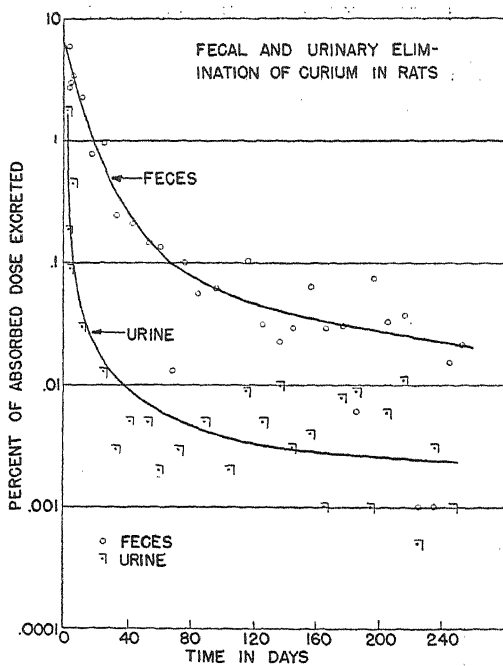
tion of curium. The excretion of curium takes place primarily by way of the digestive tract, and after most of this element stored in the liver has been released by that organ, the rate of elimination falls to a very small value, as can be seen in Text-fig. 2, which presents the change in the daily rate of excretion of curium in the urine and the feces with time. The values employed to prepare these curves were obtained from the corrected data. This, of course, produces some error due to the fact that an appreciable amount of curium is presumably absorbed from the site of injection for



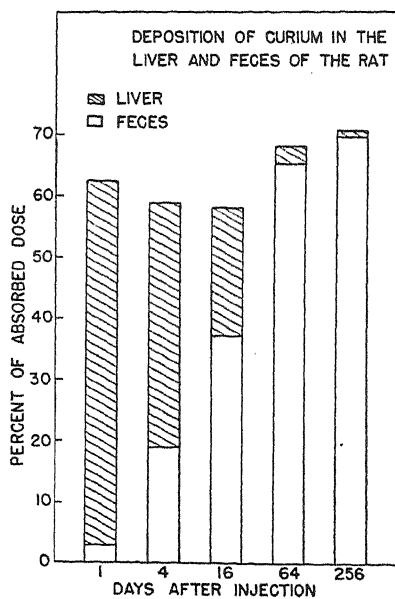
TEXT-FIG. 1.

quite some interval of time. The degree of error introduced by this manipulation of data is felt to be trivial after the first 16 days, since it is apparent the amount of curium remaining unabsorbed after the 16 day interval neither changes very significantly at the later time intervals nor does it represent more than a small proportion of the total dose given. The correlation between content of curium in the liver and the fecal excretion is shown graphically in Text-fig. 3. It is apparent that the removal of curium from the liver at the various time intervals is closely paralleled by the quantity of this element appearing in the feces.

The degree of absorption of curium from the digestive tract, following



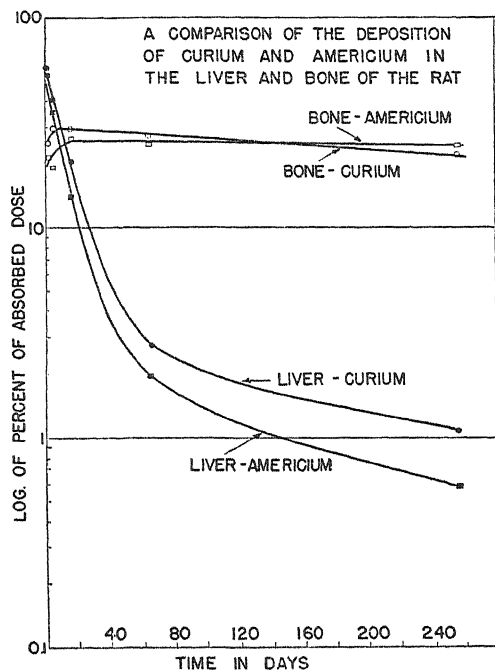
TEXT-FIG. 2.



TEXT-FIG. 3.

administration by stomach tube, was observed to be less than 0.05 per cent of the dose given to the animals.

The section of femur and its corresponding radioautograph, shown in Fig. 1, indicate that curium is deposited in the region of the periosteum, endosteum, and the trabeculae. The mineralized portion of the bone shows deposition of curium in small spotty areas. At the magnification shown in Fig. 1, it is difficult to correlate the regions in which curium has been apparently laid down in the cortical bone with histological structure.



TEXT-FIG. 4.

Fig. 2 shows a small area of cortical bone and the corresponding region of the radioautograph at a higher magnification. The deposition of curium in most cases is in the region of the small blood vessels located in the cortical bone.

#### DISCUSSION

The close similarity of the metabolic characteristics of americium to those of the lanthanum group of rare earths has already been discussed briefly. It is of interest to compare the almost identical behavior of americium and curium in the animal body. The three most striking meta-

bolic properties of these two heavy elements are their high uptake and comparatively rapid excretion by the liver, their deposition and prolonged retention by the skeleton, and the curious pattern of distribution about the small blood vessels of cortical bone.

Text-fig. 4 presents the change in content of americium and curium in the liver and bone at the five time intervals, which extend from 1 to 256 days. It can be seen that the degree of differences of their content in these two organs is small at the corresponding time intervals. In fact, it is felt that the variations noted are quite possibly within the experimental errors common to both series of studies. Thus it has been demonstrated that the behavior of these two artificially prepared radioactive elements in the animal body is essentially indistinguishable under the conditions of the experiments described in this report.

The selective deposition of curium in the region of the radiosensitive bone marrow makes it a hazardous substance should it gain entry into the body and be absorbed. It is of interest to note that its half time in the skeleton of the rat is much greater than its radioactive half life of 150 days. It would appear probable that this new element may be expected to behave in a similar manner in man.

#### SUMMARY

The fate of curium has been studied in the rat at periods of 1, 4, 16, 64, and 256 days after intramuscular administration.

About five-sixths of the curium is absorbed from the site of administration in the first 16 days after injection. Following absorption, curium is primarily deposited in the liver and skeleton. The curium present in the soft tissues of the body is eliminated primarily via the gastrointestinal tract. Curium is not absorbed to any significant degree from the gastrointestinal tract. Radioautographic studies show that curium is deposited in the region of the periosteum, endosteum, and the endosteal covering of trabecular bone, and in the region of the small blood vessels of the cortical bone. Curium is retained by the skeleton for a long period of time. The metabolism of curium and americium in the rat is very similar.

The authors gratefully acknowledge the advice and suggestions of Professor G. T. Seaborg, Professor I. Perlman, and their colleagues, and the technical assistance of Edith Steinhauß and Patricia Wallace.

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## EXPLANATION OF PLATES

## PLATE 1

FIG. 1. Photomicrograph of bone section and radioautograph of femur from rat injected with curium and sacrificed at 7 days. There is a very heavy deposit of curium in the region of trabecular bone below the epiphysis. The periosteal and endosteal surfaces show a definite accumulation of curium, and in the shaft, curium is deposited in a very spotty pattern.  $\times 7$ .

## PLATE 2

FIG. 2. Higher magnification of a small area of shaft and corresponding area of the radioautograph shown in Fig. 1. The curium deposits which are spottily distributed in the shaft appear to be associated with blood vessels.  $\times 60$ .

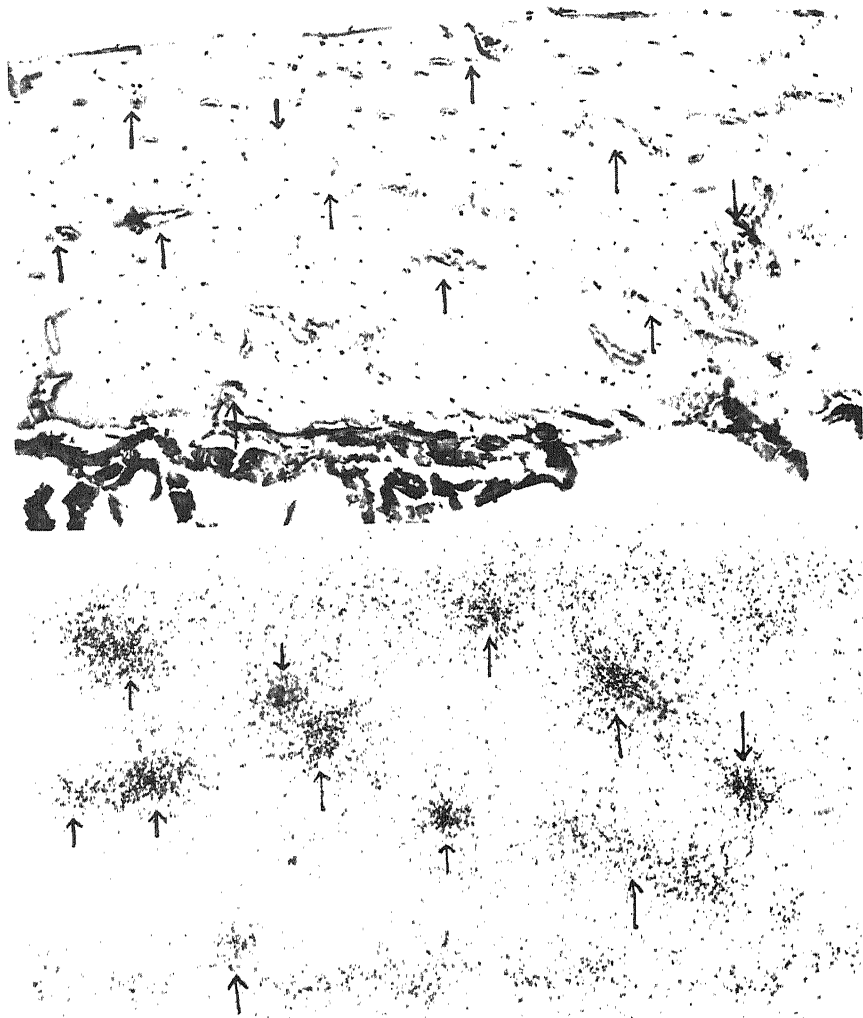






(Scott, Axelrod, and Hamilton: Curium)





(Scott, Axelrod, and Hamilton: Curium)



# A NEW COLOR TEST FOR TRYPTOPHAN AND RELATED COMPOUNDS

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(Received for publication, September 10, 1948)

It has been reported in a preliminary communication (1) that under certain conditions, at room temperature, perchloric acid converts tryptophan to a yellowish green fluorescent compound. Fluorescence was found to be particularly strong in ultraviolet light. It has now been found that a small amount of bichromate very considerably increases the sensitivity of this reaction and that the test may also be applied to certain compounds which are related to tryptophan. This reaction, however, is not given by any amino acid other than tryptophan. Tryptophan may thus be readily identified in untreated proteins.

## EXPERIMENTAL

### *Solutions*

*Perchloric Acid*—The usual commercial product, C.P., 70 to 72 per cent.

*Dichromate Solution*—10 mg. of potassium dichromate (C.P.) in 100 cc. of distilled water.

*Ferric Chloride Solution*—1 gm. of ferric chloride ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , C.P.) in 100 cc. of distilled water.

### *Color Test*

0.5 cc. of water containing 0.5 mg. of tryptophan or about 10 mg. of albumin (egg powder) or any other tryptophan-containing substance is placed in a test-tube. It is not necessary that the material to be tested shall be in solution. If certain indole derivatives are to be tested, they may be dissolved in ethyl alcohol. 3 cc. of perchloric acid and 0.1 cc. of bichromate solution are added and the contents of the tube are well mixed. A quite stable, intensely greenish yellow color forms immediately. Upon the addition of 0.1 cc. of 1 per cent ferric chloride solution, the greenish yellow color becomes deep reddish orange within a few minutes. For the detection of minute amounts of tryptophan, the addition of ferric chloride solution should be omitted and ultraviolet light should be employed.

In Table I are shown typical color reactions as given by tryptophan and a few compounds closely related to tryptophan. All substances, however,

show much less intense fluorescence when treated with perchloric acid and bichromate solution than does tryptophan. Distinct color differences are displayed by the various compounds after the addition of the ferric chloride solution to the perchloric acid-bichromate reaction mixture. The following tryptophan-containing proteins gave the color reaction: casein, albumin (egg powder), human blood serum, pepsin, and crystalline soy bean trypsin inhibitor. Gelatin (Difco) and silk (U. S. P. type A-O, Product 480, Davis and Geck) did not give the test. A 10 mg. sample of zein in 80 per cent ethyl alcohol showed only very slight fluorescence, indicating the presence of only a trace of tryptophan.

TABLE I  
*Color Reactions with Perchloric Acid-Dichromate and Ferric Chloride*

	Color after addition of perchloric acid and dichromate	Color after subsequent addition of ferric chloride
Tryptophan.....	Greenish yellow	Reddish orange
Skatole.....	Light yellow	Intense yellow
Indole .....	" " (almost colorless)	Greenish blue
" acetic acid.....	Pink	Brown

#### SUMMARY

Tryptophan and related compounds give typical color reactions with the perchloric acid test. The amino acids, glycine, alanine, leucine, isoleucine, valine, phenylalanine, tyrosine, cysteine, cystine, methionine, threonine, proline, hydroxyproline, histidine, arginine, lysine, serine, aspartic acid, glutamic acid, and *p*-aminobenzoic acid do not give the reaction.

Although certain phenols react with ferric chloride (as in the second part of this test), perchloric acid and dichromate do not give a color reaction with small amounts of phenols and the phenols do not form fluorescent compounds under similar conditions.

Cohen (2) made the remarkable observation that, when carbohydrates are heated together with tryptophan for 10 minutes at 100° in 30 per cent perchloric acid, colored condensation products form. In his reaction boiling is an essential factor. The green fluorescent substances described herein, however, form immediately at room temperature and carbohydrates do not interact.

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# PHOSPHOPROTEIN PHOSPHATASE IN MAMMALIAN TISSUES\*

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(Received for publication, June 25, 1948)

In 1946 Harris (1) demonstrated the existence in frog eggs of a new enzyme, which he designated phosphoprotein phosphatase, capable of removing inorganic phosphorus directly from phosphoproteins without preliminary proteolysis. The significance of the occurrence of phosphoproteins only in food sources for the embryo and the young (*e.g.*, casein in milk, vitellin in egg yolk, ichthulin in fish eggs) has never been completely explained. Should a parallel exist between the distribution of phosphoproteins and phosphoprotein phosphatase, this fact would be of the greatest interest in studies of normal and abnormal growth. The following report presents evidence for the existence of this enzyme in mammalian tissues, discusses some properties of the enzyme, describes a method for its assay, and gives some indication of its distribution in various tissues of the rat and in the spleen of other mammals.

## EXPERIMENTAL

*Analytical Methods*—Inorganic phosphorus analyses were performed by the method of Fiske and Subbarow (2). Protein was determined by the method of Robinson and Hogden (3). This method, however, is not very satisfactory for frog egg homogenates, due to difficulty in preparing clear biuret color solutions.

For the determination of total phosphorus and total nitrogen, a modification of the direct nesslerization method of Koch and McMeekin (4) was used.

*Biological Methods*—Unless otherwise stated, the enzyme source was rat spleen, homogenized (5) with 5 volumes of ice-cold H<sub>2</sub>O. Casein specially prepared from skim milk by the methods of Van Slyke and Bosworth (6) and Van Slyke and Baker (7) was used as substrate; it was suspended in dilute alkali and homogenized for uniform distribution. Enzyme, substrate, and all other additions were brought to pH 6.0 before use. Ordinarily 3 ml. of casein suspension were incubated with 0.5 to 1.0 ml. of tissue

\* The work described in this paper was done under contract between the Medical Division, Chemical Corps, United States Army, and the University of Chicago Toxicity Laboratory. Under the terms of the contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

homogenate and sufficient activator, inhibitor, etc., to make a final volume of 5.0 ml. Incubation was at 37°, and the reaction was stopped after 10 minutes by the addition of 20 ml. of 10 per cent trichloroacetic acid. Deproteinized tubes were kept in an ice bath for 15 minutes before filtration through Whatman No. 3 filter paper.

A few experiments were performed with frog eggs. In these cases, female frogs (*Rana pipiens*) were pithed, and the egg sacs were removed, weighed, and homogenized in 50 volumes of cold water. Deproteinization was performed as with spleen homogenates, except that the tubes were kept in the ice bath for 60 minutes before filtering. With this precaution, water-clear filtrates were always obtained.

*Evidence for Identity of Enzyme*—Several experiments were performed to demonstrate that the action of spleen homogenates in releasing inorganic phosphorus from casein is a direct one, rather than a two-stage reaction of proteolysis followed by phosphatase action. For reasons discussed later, the following experiments were performed with 0.01 M ascorbic acid in the incubating mixture.

Were inorganic phosphate being released via a pathway involving proteolytic activity, it would be expected that the addition of a large quantity of phosphorus-free protein would reduce the proteolysis of phosphoprotein simultaneously present, and hence decrease the appearance of inorganic phosphorus. The addition of dialyzed hemoglobin to spleen homogenate incubated with casein is without such effect.

Were inorganic phosphorus being released via a pathway involving the action of phosphomonoesterase, it would be expected that, if an excess of glycerophosphate were added to an incubating homogenate, the further addition of casein would be without effect. This is not the case; when casein is added to such a system, considerably more inorganic phosphorus appears.

The use of sodium fluoride, which inhibits a variety of enzymes including phosphomonoesterase, permits a more detailed analysis of the sources of solubilized nitrogen and phosphorus. The data of Table I demonstrate that, while fluoride sharply reduces the appearance of inorganic phosphorus, it is essentially without effect on the appearance of acid-soluble nitrogen, while at the same time there is no accumulation of organic phosphorus.

Serine phosphate has been shown by the work of Posternak (8), Lipmann (9), Levene and Schormüller (10), and others to be the basic phosphorus-containing unit of casein and vitellin. It would be expected that, if the release of inorganic phosphorus from casein proceeded over the two stages of proteolysis and phosphatase action, serine phosphate would serve ideally as a substrate. Spleen extracts, however, produce only small amounts of



inorganic phosphorus from phosphoserine<sup>1</sup> under conditions in which inorganic phosphorus is rapidly liberated from casein.

*Properties of Enzyme*—To determine the possible need for cofactors for the activity of phosphoprotein phosphatase, the effect of boiled tissue juice was tested. To accentuate any possible need for cofactors, the tissue homogenate for these experiments was prepared with 25 volumes of cold water instead of the usual 5 volumes, and the incubation was conducted for 1 hour instead of the usual 10 minutes. The tissue juice used was prepared from rabbit liver and spleen, minced, boiled, filtered, and brought to pH 6.0. In the absence of ascorbic acid, boiled tissue juice caused a 46 per cent increase in the appearance of inorganic phosphorus. The addition of 0.01

TABLE I  
*Effect of NaF on Appearance of Acid-Soluble Nitrogen and Phosphorus from Casein by Rat Spleen Extract*

300 mg. casein..... 0.01 M NaF.....	+	+	-	-
	γ	γ	γ	γ
Inorganic P found.....	103	44	36	31
“ “ formed from casein*.....	65	11		
Total acid-soluble P found.....	154	96	80	83
“ “ “ formed from casein*..	70	9		
“ “ N found.....	263	252	175	174
“ “ “ formed from casein*..	50	40		

\* Corrected for initial casein content of 2 γ of inorganic P, 4 γ of total acid-soluble P, and 38 γ of total acid-soluble N.

M ascorbic acid caused a 150 per cent increase in inorganic phosphorus, but the addition of boiled tissue juice was without effect in the presence of ascorbic acid.

Table II indicates the effect of various salts on the appearance of inorganic phosphorus, both in the presence and in the absence of ascorbic acid.

To test the possibility of maintaining tissues in the frozen state before assay, a rat was sacrificed, one-half of its spleen was dropped into a freezing mixture of dry ice and ether, and the other half was immediately assayed, as described below, for phosphoprotein phosphatase activity. Approximately an hour later, the frozen half of the spleen was thawed and similarly assayed. The two assay figures agreed within 2 per cent.

Rat spleen homogenate was brought to pH 6.0 and divided into two portions. One part was dialyzed for 2 hours against several changes of ice-

<sup>1</sup> Thanks are due to Dr. Thomas B. Coolidge for a sample of barium phosphoserine.

cold distilled water in the refrigerator; the other part was merely maintained in an ice bath for 2 hours. At the end of this time, both were assayed for enzyme activity. The two assay figures agreed within 6 per cent.

TABLE II

*Effect of Inorganic Salts on Appearance of Inorganic Phosphorus from Casein*

In Experiments 1 to 4 the results are expressed in micrograms as the difference between inorganic phosphorus concentrations before and after incubation; in Experiments 5 to 7, as total inorganic phosphorus found at the end of incubation. Salts were present in a final concentration of 0.02 M in Experiments 1 and 2 and in a final concentration of 0.01 M in Experiments 3 to 7.

Experiment No.	No ascorbic acid											0.01 M ascorbic acid	
	No salts	MgSO <sub>4</sub>	BaCl <sub>2</sub>	CaCl <sub>2</sub>	MnCl <sub>2</sub>	KCl	ZnCl <sub>2</sub>	FeSO <sub>4</sub>	NiSO <sub>4</sub>	CoCl <sub>2</sub>	CuCl <sub>2</sub>	No salts	MgSO <sub>4</sub>
1	21	29		29			19						
2	41		50		51	40		42	44	45	6		
3	24	46			42								
4	61	69											
5	162	174											
6												201	195
7	35	40										69	69

TABLE III

*Effect of Oxidizing and Reducing Agents on Liberation of Inorganic Phosphorus from Casein*

Experiment No.	Tissue	Inorganic phosphorus liberated from casein in presence of							
		None	0.01 M H <sub>2</sub> O <sub>2</sub>	Ascorbic acid				0.01 M cysteine	0.01 M KCN
				0.0001 M	0.001 M	0.005 M	0.01 M		
		γ	γ	γ	γ	γ	γ	γ	γ
1	Frog eggs	119						247	
2	" "	219	135				307		
3*	Rat spleen	81	24				274		
4	" "	144		144	170		212	196	208
5	" "					198	214		
6	" "				208		266		
7	" "	86	21				185		

\* The values in Experiment 3 are corrected for inorganic phosphorus appearing in the absence of casein; other experimental data do not include this correction.

Table III presents data indicating the strong influence of oxidizing and of reducing agents on the release of inorganic phosphorus. Particularly to be noted are the facts that (a) activation by reducing agents and inhibition

by oxidizing agents are characteristic of both the enzyme in frog eggs and that in rat spleen, (b) activation is a common property of several reducing agents, and (c) the degree of activation is dependent on the concentration of the reducing agent.

*Method of Assay*—Without the addition of ascorbic acid or other reducing agent, the enzyme activity is not only not maximal, but it is also not linear; *i.e.*, the inorganic phosphorus liberated from the casein is not proportional to the amount of tissue extract added. That 0.01 M ascorbic acid corrects this difficulty is shown by the data of Table IV. Concentrations of ascorbic acid up to 0.01 M in the enzyme assay tubes have been found to be without effect on the final Fiske and Subbarow phosphorus determination; higher

TABLE IV  
*Effect of 0.01 M Ascorbic Acid on Linearity of Enzyme Assay*

Experiment No.	Inorganic phosphorus released from casein per ml. homogenate						
	No ascorbic acid				0.01 M ascorbic acid		
	0.2 ml. homogenate	0.5 ml. homogenate	1.0 ml. homogenate	2.0 ml. homogenate	0.2 ml. homogenate	0.5 ml. homogenate	1.0 ml. homogenate
	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$
1	85	94	124	171			
2	95		145				
3	55		81		290		274
4					410		435
5					375		362
6						156	155
7						114	110

All figures are corrected for initial inorganic phosphorus of all components and for inorganic phosphorus formed in the absence of casein.

concentrations than this, however, cause analytical artifacts, presumably by the reduction of molybdate.

Because of the effect of 0.01 M ascorbic acid, ascorbic acid of this concentration was used routinely in all assays. As an additional check on the linearity of the assay, each tissue assayed was tested in two concentrations, rather than in duplicate volumes.

Control tubes provided the necessary data as to inorganic and total acid-soluble phosphorus and total acid-soluble nitrogen initially present in, and formed from, the tissue homogenate itself. In addition, each lot of casein prepared was analyzed for these three constituents, and the proper corrections were applied to the assay figures.

*Phosphoprotein Phosphatase Distribution in Other Animal Tissues*—Table V presents the results of assay of various rat tissues by the method described

above. All rats were fasted for 24 hours before sacrifice. Table VI presents the results of assay of the spleen of the mouse, the hamster, the guinea pig, and the rabbit.

TABLE V  
*Assay of Phosphoprotein Phosphatase in Rat Tissues*

Experiment No.	Tissue	Released from 300 mg. casein by 1 gm. tissue			Protein per gm. tissue
		Inorganic P	Total acid-soluble P	Total acid-soluble N	
		mg.	mg.	mg.	mg.
1	Spleen	0.85	0.84	1.08	124
	Liver	0.30	0.30	1.06	154
	Kidney	0.39	0.42	1.77	100
	Heart	0.15	0.14	0.01	102
2	Spleen	1.16	1.17	1.17	
	Liver	0.27	0.35	0.71	
	Intestine	0.29	0.35	0.72	
3	Spleen	0.45	0.46	0.82	144
	Stomach	0.10	0.26	5.14	47
	Testis	0.21	0.26	0.20	48
	Muscle	0.05	0.07	0.08	138
4	Spleen	0.56	0.56	0.79	138
	Liver	0.27	0.27	0.81	147
	Brain	0.32	0.36	0.12	67
	Lung	0.17	0.16	0.11	113

TABLE VI  
*Phosphoprotein Phosphatase in Spleen of Species Other Than Rat*

Species	Released from casein by 1 gm. tissue			Protein per gm. tissue.
	Inorganic P	Total acid-soluble P	Total acid-soluble N	
	mg.	mg.	mg.	mg.
Mouse.....	0.76	0.77	1.35	86
Hamster.....	0.46	0.45	0.78	146
Guinea pig.....	1.37	1.22	0.68	86
Rabbit.....	0.66	0.65	3.60	94

#### DISCUSSION

The enzymic decomposition of casein and other phosphoproteins has been frequently investigated in the past. Biffi (11), using pancreatic extracts, and Travia and Veronese (12, 13), using an alkaline phosphatase from ox spleen, claimed the liberation of inorganic phosphorus from casein. Harris (1), using homogenates of frog eggs, was the first to demonstrate the existence of an enzyme specifically catalytic for this reaction.

On the other hand, most authors have either been unable to demonstrate such a reaction, or have used combinations of proteinases and phosphatases, or have used extracts in which it was felt that proteolytic activity was preceding phosphatase action. Among this work is that of Salkowski (14), Plimmer and Bayliss (15), Kay (16), Rimington and Kay (17), Rimington (18, 19), Sadamitsu (20), and Schmidt and Thannhauser (21).

The demonstration of the existence of the enzyme phosphoprotein phosphatase in crude extracts depends primarily upon distinguishing between (a) the one-stage reactions whereby inorganic phosphorus is released directly from phosphoprotein and (b) the two-stage reaction of proteolysis followed by phosphatase action. The ideal demonstration would be the establishment of such conditions that inorganic phosphorus is released without the simultaneous appearance of acid-soluble nitrogen. This has not yet been achieved; a definite but somewhat erratic amount of non-protein nitrogen has always appeared in these experiments. It has therefore been necessary to use only indirect evidence, which, however, seems quite conclusive. In particular, the action of fluoride in (a) decreasing phosphorus release, (b) not affecting appearance of non-protein nitrogen, and (c) not causing an accumulation of organic acid-soluble phosphorus seems final proof in itself.

Although the complete proof of identity of enzyme has not been established for all tissues tested, there seems little reason to doubt that the assay is actually measuring phosphoprotein phosphatase in all the tissues examined, with the probable exception of rat stomach, in which the total acid-soluble phosphorus released is distinctly greater than the inorganic phosphorus, and in which a considerable amount of nitrogen becomes acid-soluble.

A further point of interest lies in the action of boiled tissue juice and inorganic salts. In all cases, the small but definite activating effect of magnesium ions or of tissue juice disappears when reducing agent is added. It is possible that the  $Mg^{++}$  or tissue juice actually activates some other enzyme system which in turn, and in the absence of exogenous reducing agent, tends to provide the reducing atmosphere which activates phosphoprotein phosphatase.

#### SUMMARY

1. Evidence is presented for the existence in mammalian tissues of the enzyme phosphoprotein phosphatase, which hydrolyzes inorganic phosphorus from phosphoproteins without preceding proteolysis.

2. The phosphoprotein phosphatase activity in the absence of a reducing agent is increased by boiled tissue juice or by magnesium, manganese, barium, or calcium ions. In the presence of 0.01 M ascorbic acid, neither boiled tissue juice nor magnesium ions have any effect. In the absence of

ascorbic acid, only negligible effects, if any, are shown by  $K^+$ ,  $Fe^{++}$ ,  $Ni^{++}$ ,  $Co^{++}$ , or  $Zn^{++}$  ions, while 0.02 M  $CuCl_2$  strongly inhibits the reaction.

3. A method of assay for the enzyme is given, based on the use of purified casein as a substrate, a water homogenate of the tissue as the enzyme source, and ascorbic acid as an activator.

4. A survey has been made of the distribution of the enzyme in the various organs of the rat, and it is shown that this enzyme also exists in the spleen of the mouse, the rabbit, the guinea pig, and the hamster.

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## RAPID METHOD FOR THE DETERMINATION OF FAT IN FECES\*

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(Received for publication, August 10, 1948)

Study of fat absorption in a patient requires the determination of the fat content of feces. Quantitative chemical methods available for such determinations are too laborious to be practical for routine analyses. Estimation of the fat content of feces by microscopic examination, practised by many clinicians, is unreliable; this can be demonstrated by comparing the results of microscopic determinations with those of chemical analysis. The results of chemical methods expressing the fat content of a random sample of feces as percentage of dry matter are equivocal. Goiffon (7) and Frazer *et al.* (2) pointed out that the percentage of dry matter in feces is not necessarily constant. It may be quite variable in feces from patients with metabolic disorders or faulty absorption. The observations of Frazer and Goiffon were also confirmed in this laboratory.

Balance studies are the conventional means of evaluating certain aspects of metabolism of nitrogen, calcium, etc. Only a few publications report such studies of fat absorption; yet it seems the only exact method of investigation. Estimation of fat balance is carried out as follows: The patient is kept on a diet containing a known amount of fat. The feces excreted in 24 hours are collected accurately. In a mixed sample of these feces, fat, if necessary differentiated into split and unsplit fat, is determined quantitatively. The total amount of fat consumed in 24 hours, less the amount of excreted fat, represents the amount of retained fat. This may be expressed as per cent of the amount of fat consumed. Since intestinal motility is variable, it is advisable to determine the fat balance on several consecutive days. The frequency of such determinations depends upon the magnitude of the daily fluctuations.

The determination of the fat content of feces may be carried out before or after drying of the feces. There are various objections to the latter method: (1) During drying, conversions may take place by which the ratio of split fat to unsplit fat could be changed; (2) during drying, volatile fatty acids may evaporate; (3) drying takes a long time. These objections are eliminated by the determination of fat in wet feces. Therefore, we

\*The data in this paper are taken from the first part of a thesis submitted by J. H. van de Kamer in partial fulfilment of the requirements for the degree of Doctor of Philosophy, University of Utrecht, The Netherlands.

have sought a simple method for the determination of fat in wet feces which also meets the requirements of clinical investigation.

Von Liebermann and Székely (13), Folin (3), Polenske (16), Saxon (18), Sonntag (19), Holt, Courtney, and Fales (9), Fowweather (4, 5), Rosenfeld (17), Muller (14), Tidwell and Holt (20), Gephart and Csonka (6), Nielsen (15), Kumagawa and Suto (12), Inaba (10) (method modified by Coiffon (7)), and others have published methods for the determination of fat in wet feces. They depend upon various principles and generally give correct results. Although these methods require less time than those in which feces are first dried, they are still quite time-consuming. Applying the principles published by von Liebermann and Székely (13) and by Saxon (18), we elaborated two methods by which the fat content of feces can be determined simply and rapidly.

### *Principle of Methods*

Feces are saponified according to the procedure of von Liebermann and Székely (13) with concentrated potassium hydroxide in ethanol, giving a solution which contains the soaps derived from the neutral fats and the fatty acids and also the soaps which were originally present in the stool. By adding HCl to the alkaline solution, the fatty acids are liberated. Ethanol is then added and the fatty acids are extracted with petroleum ether. The concentration of ethanol is so chosen that, after shaking the mixture, the petroleum ether and the acid ethanol layers separate quickly; this is expedited by adding NaCl and a small amount of amyl alcohol. Separation is complete after 5 to 10 minutes. In an aliquot sample of the petroleum ether layer the fatty acids are titrated with alkali, with thymol blue as indicator. This method of determining the total fat content requires 35 minutes (Method A).

To determine split and unsplit fat separately, the sample of feces is not treated with alkali, but is boiled for 1 minute with diluted hydrochloric acid in order to convert the soaps into free fatty acids, according to the method of Saxon (18). After addition of ethanol, NaCl, and amyl alcohol, the solution is extracted with petroleum ether. An aliquot sample of the petroleum ether layer is evaporated to dryness and the free fatty acids are titrated with 0.1 N isobutyl alcoholic KOH. Thereupon an excess of the same solution is added and the unsplit fat is saponified by boiling. The excess of alkali is titrated with 0.1 N HCl and thymol blue as indicator (Method B).

### *Method A; Determination of Total Fat Content*

#### *Reagents—*

*Ethanol, 96 per cent, containing 0.4 per cent amyl alcohol.*



*Ethanol, 96 per cent, neutral to thymol blue.*

*KOH, 33 per cent.*

*HCl, 25 per cent, specific gravity 1.13.*

*Petroleum ether, boiling point 60–80°, or 40–60°. When evaporated to dryness, this must leave no residue which can be titrated or saponified with alkali.*

*NaOH, 0.1 N.*

*Thymol blue, 2 per cent, in 50 per cent ethanol.*

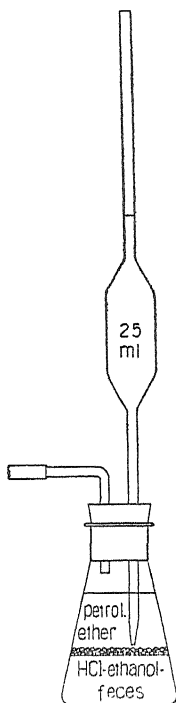


FIG. 1. Erlenmeyer flask with pressure pipette to remove aliquot of petroleum ether.

#### *Apparatus—*

150 ml. Erlenmeyer flasks with wide mouths (high model), each provided with a rubber stopper and reflux condenser.

25 ml. pipette, fitted into the flask as illustrated in Fig. 1. With this apparatus the petroleum ether solution is brought into the pipette by blowing and evaporation is prevented.

50 ml. pipette.

5 ml. micro burette.

*Procedure*—About 5 gm. of feces are weighed in a 150 ml. Erlenmeyer flask. After adding 10 ml. of 33 per cent alkali and 40 ml. of ethanol containing 0.4 per cent amyl alcohol, the mixture is boiled for 20 minutes under a reflux condenser, and then thoroughly cooled. 17 ml. of 25 per cent HCl are added, a graduated cylinder being used, after which the mixture is again cooled. Exactly 50 ml. of petroleum ether are then added, and the flask is closed with a rubber stopper and shaken vigorously for 1 minute. After complete separation, 25 ml. of the petroleum ether layer are transferred into a small Erlenmeyer flask by using the pressure pipette.

After addition of a piece of filter paper, the petroleum ether is evaporated and 10 ml. of neutral ethanol are added. The fatty acids are titrated with 0.1 N NaOH from a micro burette, with thymol blue as indicator, until the yellow color begins to change. If 0.1 N isobutyl alcoholic KOH is available (see Method B), it is possible to titrate the fatty acids directly in the petroleum ether without previous evaporation.

*Calculation*—For the present, the calculations are carried out according to Goiffon (7), assuming an average molecular weight of 284 for fatty acids.<sup>1</sup> Hence

$$\frac{A \times 284 \times 1.04 \times 2 \times 100}{10,000Q} = 5.907 \frac{A}{Q} = \text{fatty acids in gm. per 100 gm. feces}$$

in which  $A$  = the ml. of 0.1 N alkali used in titration, and  $Q$  = the gm. of feces taken for analysis. The factor 1.04 must be used as the petroleum ether layer increases 1 per cent in volume when shaken with alcoholic hydrochloric acid and because 3 per cent of the amount of fatty acids remains in solution in the acid alcoholic layer. Corrections for evaporation of the petroleum ether layer and for volume increase due to fatty acids dissolved in it may be neglected. That the factor used is correct was demonstrated by the fact that the same fat content is found when the solution is extracted quantitatively with petroleum ether and when an aliquot is used as described above.

#### *Method B; Determination of Fatty Acid and Neutral Fat Separately*

##### *Reagents—*

*HCl*, 2.5 per cent, specific gravity 1.013, to which 250 gm. of NaCl per liter are added.

*Ethanol*, 96 per cent, containing 0.4 per cent amyl alcohol.

*Ethanol*, 96 per cent, neutral to thymol blue.

*Petroleum ether*, boiling point 60–80°, or 40–60°, as described above.

*Isobutyl alcoholic KOH*, 0.1 N. Isobutyl alcohol is boiled for 3 hours

<sup>1</sup> Another paper deals with the determination of the molecular weights.

with 100 gm. of NaOH per 5 liters. It is then distilled and the fraction that comes over between 105–108° is collected. To 5 liters of this solution are added 15 gm. of concentrated 50 per cent KOH solution diluted with 20 ml. of methyl alcohol. The KOH solution is prepared by dissolving solid KOH in an equal amount of water; after standing for several days the clear solution is siphoned off. The diluted isobutyl alcoholic KOH is titrated with 0.1 *N* hydrochloric acid, with thymol blue as indicator, until the yellow color begins to change.

*Hydrochloric acid, 0.1 N.*

*Thymol blue, 2 per cent, in 50 per cent ethanol.*

*Apparatus—*

Cylindrical tubes of 30 cm. length and 4 cm. diameter, provided with 50 cm. reflux condensers with ground joints and glass stoppers.

25 ml. pipette. This is fitted into the cylindrical tubes by means of a stopper, as illustrated in Fig. 1.

100 ml. Erlenmeyer flasks provided with glass bulbs as reflux condensers.

50 ml. pipette.

5 ml. micro burette.

*Procedure—*About 5 gm. of feces are weighed in a cylindrical tube. After addition of 22 ml. of HCl solution containing NaCl and some grains of pumice, the mixture is boiled for 1 minute under the reflux condenser. The tube is then thoroughly cooled. 40 ml. of 96 per cent ethanol containing 0.4 per cent amyl alcohol are added by means of a graduated cylinder, and exactly 50 ml. of petroleum ether by means of a pipette. After this the tube is closed with the glass stopper and vigorously shaken for 1 minute.

Separation may be facilitated by occasional rotation of the tube. When separation is completed, 25 ml. of the petroleum ether layer are transferred into a 100 ml. Erlenmeyer flask with the help of the pressure pipette (Fig. 1). After addition of a small piece of filter paper, the petroleum ether is evaporated; 2 ml. of neutral ethanol are added and the free fatty acids are titrated with 0.1 *N* isobutyl alcoholic KOH in the micro burette, with thymol blue as indicator, until the yellow color begins to change.

After addition of 10 ml. of 0.1 *N* isobutyl alcoholic KOH, the solution is boiled gently for 15 minutes, the flask being provided with a glass bulb as a reflux condenser.

To the hot solution 10 ml. of neutral ethanol are added, after which the excess alkali is titrated immediately with 0.1 *N* hydrochloric acid until the blue color of the indicator turns yellow.

*Calculation—*Assuming a molecular weight for fatty acids of 284 and for fat of 297,

$$\frac{A \times 284 \times 1.04 \times 2 \times 100}{10,000Q} = 5.907 \frac{A}{Q} = \text{fatty acids in gm. per 100 gm. feces}$$

$$\frac{(B - C) \times 297 \times 1.01 \times 2 \times 100}{10,000Q} = 5.999 \frac{(B - C)}{Q} = \text{neutral fat in gm. per 100 gm. feces}$$

in which  $A$  = the ml. of 0.1 N alkali used in titration of the fatty acids,  $B$  the ml. of 0.1 N HCl used in the blank titration of 10 ml. of isobutyl alcoholic KOH,  $C$  the ml. of 0.1 N HCl used in the titration of fat, and  $Q$  the gm. of feces taken for analysis. The factor 1.04 for fatty acid is explained above; for neutral fat the factor is 1.01, since in this case only the increase in volume of the petroleum ether layer has to be corrected.

### *Remarks*

1. After boiling with HCl the solution must be cooled thoroughly; otherwise, after addition of alcohol, esterification of the fatty acids may take place.

2. The use of long cylindrical tubes in Method B is necessary to avoid loss of volatile fatty acids.

3. In order to prevent irregular boiling during evaporation of the petroleum ether, a small piece of filter paper is used. No pumice must be used, as this absorbs fatty acids.

4. It is not necessary to evaporate the petroleum ether quantitatively, as small amounts of this solvent have no influence on the titration nor on the saponification.

5. Saponification of the fatty acids is carried out according to Kolthoff (11) with 0.1 N isobutyl alcoholic KOH. Compared with the usual 0.5 N ethanolic KOH it has two advantages. In the first place, the boiling point of the isobutyl alcoholic solution is about 100° instead of 80° as in the case of ethanol; for this reason the saponification time is reduced considerably. Secondly, the accuracy of the titration is increased because it is possible to use 0.1 N isobutyl alcoholic KOH instead of the usual 0.5 N ethanolic KOH.

6. For the extraction of the acid alcoholic solution with petroleum ether, 60 per cent alcohol appears to be most suitable. With lower concentrations, emulsions are easily formed, while at higher concentrations too much fatty acid remains dissolved in the alcoholic layer.

7. The extraction is complete after 1 minute; no more fat is extracted after shaking the mixture for 2, 3, 4, 5, or 10 minutes.

8. Usually phenolphthalein is used as indicator in fatty acid titration. In the yellow-colored petroleum ether solution, however, the change in color of thymol blue from yellow to green is much more evident than the

change of phenolphthalein from yellow to reddish yellow. The two indicators have the same pH range, *viz.* from pH 8 to 10.

9. Addition of NaCl and amyl alcohol to the acid alcoholic solution enhances the separation of the alcoholic and the petroleum ether layer.

10. Titration of the fatty acid was preferred to weighing because it is quicker and because unsaponifiable matter is excluded at the same time.

11. It appeared that fatty acids and fat added to feces could be recovered separately with a relative error of 2 per cent.

### *Comparison of Various Methods*

The results of determinations, carried out according to Methods A and B, were compared with determinations according to other well known methods.

*Method of Weibull (21, 22)*—About 5 gm. of feces are boiled for 1 hour with 100 ml. of 1.5 N HCl, filtered through a wet filter, and washed with water until all acid has disappeared (neutral reaction to Congo red paper). The filter, after drying overnight at 40°, is extracted with petroleum ether, according to Berntrop (1). The petroleum ether is evaporated and the residue is titrated according to our Method A.

*Method of Gorter and de Graaff (8)*—About 5 gm. of feces are mixed with 2 ml. of 25 per cent HCl and 10 gm. of sand, evaporated to dryness on a water bath, and extracted according to the procedure of Berntrop (1).

*Method of Fowweather (4)*—About 3 gm. of feces are mixed in a mortar with 2 ml. of 25 per cent HCl and 20 gm. of desiccated CaSO<sub>4</sub>, and the mixture is stirred to dryness. After standing for 30 minutes it is stirred again and extracted according to the procedure of Berntrop (1). As it appeared that the results were very irregular, the procedure was modified as follows: After stirring to dryness, the mixture was dried at 40° overnight and afterwards extracted.

*Method of Muller (14)*—About 5 gm. of feces are mixed with 2 ml. of 25 per cent HCl; then the mass is stirred with some acetone. The acetone is filtered through a Büchner funnel and the residue is washed with acetone until a fine dry powder remains. After evaporation of the acetone, the residue is extracted several times with petroleum ether. The petroleum ether is filtered through a crucible with fritted disk and a layer of cotton wool into a weighed flask. After evaporation the residue is dried at 100° for 1 hour and weighed, or, if possible, titrated. If the fat contains impurities, it may be too dark for titration. The results are given in Table I.

The following conclusions may be drawn from Table I: (a) After acid treatment of feces (our Method B) the same amount of fatty acids and fat is found as after alkali treatment (our Method A). (b) The first five methods give practically the same results. (c) The method of Muller is

not exact, the extracts being too dark for titration; moreover, the values obtained by weighing are irregular and not correct.

TABLE I  
*Comparison of Results of Various Methods*

Sample No.	Various methods	Duplicate analyses, per cent of fresh feces		
		Fatty acids	Fat	Fatty acids + fat
1	Our Method A			6.2, 6.2
	“ “ B	5.8, 5.5	0.51, 0.51	6.3, 6.0
	Weibull	5.5, 5.6	0.64, 0.64	6.1, 6.2
	Gorter and de Graaff	5.6, 5.6	0.68, 0.58	6.3, 6.2
	Fowweather (modified)	5.6, 5.5	0.62, 0.58	6.2, 6.1
	Muller (titrated)	5.2, 5.3	0.70, 1.10	5.9, 6.4
2	“ (weighed)			6.5, 7.0
	Our Method A			5.6, 5.6
	“ “ B	5.0, 5.0	0.70, 0.60	5.7, 5.6
	Weibull	5.0, 5.0	0.24, 0.36	5.3, 5.4
	Gorter and de Graaff	4.9, 4.9	0.51, 0.53	5.4, 5.4
	Fowweather (modified)	5.0, 4.9	0.48, 0.53	5.5, 5.4
3	Muller (titrated)	5.1, 5.2	1.30, 1.50	6.4, 6.7
	“ (weighed)			6.0, 6.2
	Our Method A			10.5, 10.5
	“ “ B	9.1, 9.1	1.4, 1.5	10.5, 10.6
	Weibull	9.2, 9.1	0.9, 0.9	10.1, 10.0
	Gorter and de Graaff	9.2, 9.2	1.1, 1.0	10.3, 10.2
4	Fowweather (modified)	8.9, 9.1	1.2, 1.3	10.1, 10.4
	Muller (end-point not visible)			
	“ (weighed)			11.7, 10.3
	Our Method A			5.6, 5.6
	“ “ B	4.9, 4.9	0.8, 0.8	5.7, 5.7
	Weibull	5.0, 4.9	0.5	5.4
	Gorter and de Graaff	4.8, 5.0	0.6, 0.6	5.4, 5.6
	Fowweather (modified)	4.8, 4.8	0.5, 0.6	5.3, 5.4
	Muller (end-point not visible)			
	“ (weighed)			6.6, 6.7

#### SUMMARY

Methods for the determination of the total amount of fat in feces and of the relative proportions of free and combined fatty acids are described.

They are based on the principle that fatty acids and fat can be extracted almost quantitatively with petroleum ether from an acidic, alcoholic solution of about 60 per cent ethanol, saturated with NaCl and containing a small amount of amyl alcohol.

By these methods the fat in feces can be measured within 35 to 45 minutes with an error not exceeding 2 per cent.

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# A NEW SYNTHESIS OF CYTOSINE AND 5-METHYLCYTOSINE

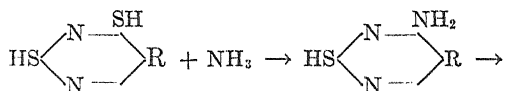
BY GEORGE H. HITCHINGS, GERTRUDE B. ELION, ELVIRA A. FALCO,  
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(Received for publication, September 16, 1948)

The chief difficulty in the way of a fruitful synthesis of cytosine from uracil is the essentially equal reactivities of chlorine atoms and of ethoxyl groups in the 2 and 4 positions of the pyrimidine nucleus. The reaction of 2,4-dichloropyrimidine with ammonia leads to a mixture of chloroaminopyrimidines (1) which is separable only after transformation to the methoxyaminopyrimidines. The reaction of dichloropyrimidine with sodium ethoxide has been offered as an alternative (2), since the diethoxypyrimidine so formed, on further treatment with sodium ethoxide, is converted to a mixture of ethoxyhydroxypyrimidines, the sodium salts of which are separable. Since the ethoxyl groups are replaceable by amino groups, the individual isomers can be converted to cytosine and isocytosine respectively. Both methods have been used in preference to the original method of Wheeler and Johnson (3, 4) which proceeds from 2-ethylmercapto-4-hydroxypyrimidines via chlorination, amination, and subsequent hydrolysis of the ethylmercapto grouping.

The discovery that the 4-thiol group of 2,4-dithiolpyrimidines is much more reactive toward ammonia and amines than is the 2-thiol group<sup>1</sup> has opened up a new route to the synthesis of 4-aminopyrimidine derivatives. The ready availability of dithiolpyrimidines from thiol-, hydroxy-, and

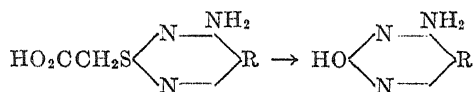


(I) R = H

(II) R = CH<sub>3</sub>

(III) R = H

(IV) R = CH<sub>3</sub>



(V) R = H

(VI) R = CH<sub>3</sub>

(VII) R = H

(VIII) R = CH<sub>3</sub>

alkylmercaptopyrimidines (5) allows a considerable latitude in the choice of starting materials. From dithiouracil (I) or dithiothymine (II) the 2-

<sup>1</sup> Russell, P. B., Elion, G. B., Falco, E. A., and Hitchings, G. H., to be published.

thiol-4-aminopyrimidines (III, IV) are obtained in good yield, and the subsequent hydrolysis of the carboxymethylthiopyrimidines (V, VI) to the 2-hydroxy-4-aminopyrimidines (VII, VIII) proceeds without difficulty. An over-all yield of 60 per cent reckoned from dithiouracil or 40 per cent from thiouracil is obtainable by this method. This may be contrasted with the 20 per cent yield from uracil obtainable by the method of Hilbert and Johnson (1).

#### EXPERIMENTAL

*2-Thiol-4-aminopyrimidine*—Dithiouracil (2 gm.) was dissolved in 20 ml. of concentrated ammonium hydroxide and the solution heated in a sealed tube at 100° for 16 hours. On cooling, 2-thiol-4-aminopyrimidine (1.1 gm.) separated as long, colorless needles. Evaporation of the mother liquors to a small volume gave another 0.5 gm. (total yield, 91 per cent). The product was washed with concentrated ammonium hydroxide to remove any unchanged dithiouracil and recrystallized from 70 ml. of water. It formed long, colorless needles, m.p. 285–290° (decomposition), after darkening at about 250°.

$C_4H_5N_3S$ . Calculated, C 37.8, H 3.9; found, C 38.2, H 4.2

*2-Carboxymethylthio-4-aminopyrimidine*—The above thiolaminopyrimidine (1 gm.) was refluxed with a solution of 0.7 gm. of chloroacetic acid in 7 ml. of water for 45 minutes. After cooling, the solution was neutralized with 2.5 N sodium hydroxide solution, acidified with acetic acid, and allowed to stand. Platelets separated (1.1 gm., 80 per cent) which, after recrystallization from a mixture of alcohol and ether, melted at 220° (decomposition), after darkening above 200°.

$C_6H_7N_3O_2S$ . Calculated, C 38.9, H 3.8; found, C 39.3, H 3.8

*Cytosine (2-Hydroxy-4-aminopyrimidine)*—The above carboxymethylthiopyrimidine (1.05 gm.) was dissolved in 10 ml. of concentrated hydrochloric acid and refluxed gently for 2 hours. After evaporation to dryness on the steam bath, the residue was treated with dilute hydrochloric acid and evaporated to dryness again. On the addition of 5 ml. of water and 1 ml. of concentrated ammonium hydroxide shiny platelets were formed (0.5 gm., 83 per cent). After recrystallization from 10 ml. of water, the compound melted at 312° (decomposition), with darkening above 290°.

$C_4H_5N_3O$ . Calculated, C 43.2, H 4.5, N 37.8; found, C 43.4, H 4.4, N 37.7

The substance gave a picrate decomposing at 333°.

The ultraviolet absorption spectra of this specimen of cytosine agree with the published values of Stimson and Reuter (6), giving in 0.1 N hydrochloric acid solution a maximum at 275 m $\mu$ ,  $E_m = 10,450$ ; in 0.1 N sodium hydroxide solution a maximum at 281 m $\mu$ ,  $E_m = 7000$ . In glycine-sodium

hydroxide buffer at pH 11, the absorption is almost identical with that in unbuffered aqueous solution (maximum at 267 m $\mu$ ,  $E_m$  = 6150).

*2-Thiol-4-amino-5-methylpyrimidine*—Dithiothymine (2.5 gm.) was dissolved in 50 ml. of concentrated ammonium hydroxide and heated at 100° for 16 hours in a sealed tube. After cooling, the white needles were filtered off (1.5 gm.). Evaporation of the mother liquors yielded another 0.2 gm., giving a total yield of 95 per cent. The product was purified by washing the crystals with ammonium hydroxide and recrystallization from water, m.p. 273–274° (decomposition).

$C_6H_7N_3S$ . Calculated, C 42.6, H 5.0; found, C 42.3, H 4.9

*2-Carboxymethylthio-4-amino-5-methylpyrimidine*—1 gm. of 2-thiol-4-amino-5-methylpyrimidine was refluxed with a solution of 0.67 gm. of chloroacetic acid in 10 ml. of water for 30 minutes. On cooling, a small amount of yellow material separated which was removed by filtration. The filtrate was neutralized with 2.5 N sodium hydroxide solution and brought back to pH 6 with acetic acid. The product separated as colorless needles (1.1 gm., 79 per cent). After recrystallization from a 1:1 mixture of alcohol and ether, it melted at 193–194°.

$C_7H_9O_2N_3S$ . Calculated, C 42.2, H 4.5; found, C 42.4, H 4.5

*5-Methylcytosine (2-Hydroxy-4-amino-5-methylpyrimidine)*—The above carboxymethylthiopyrimidine (1 gm.) was refluxed for 2 hours with 5 ml. of concentrated hydrochloric acid. On neutralization of the reaction mixture with ammonium hydroxide, the basic hydrochloride of 5-methylcytosine,  $(C_6H_7ON_3)_5 \cdot 3H_2O \cdot 2HCl$  (4), separated and was filtered off (0.6 gm., 67 per cent). This product was converted to the monohydrochloride by solution in 2.5 N hydrochloric acid and precipitation with acetone and recrystallized by the same procedure. It formed colorless platelets, m.p. 299–301° (decomposition), after sintering at about 280°.

$C_6H_7ON_3 \cdot HCl$ . Calculated. C 37.2, H 4.3, N 26.0, Cl 22.0  
Found. " 37.1, " 4.6, " 26.1, " 22.1

The picrate melts at 290–291° (decomposition).

The ultraviolet absorption spectra of this 5-methylcytosine at pH 1.0 and at pH 11.0 are given in Fig. 1. These curves are identical with those given by an authentic specimen of 5-methylcytosine which was prepared by the method of Wheeler and Johnson (4).

*5-Methylcytosine from 2-Thiol-4-amino-5-methylpyrimidine*—In an early experiment, 5-methylcytosine was prepared from 2-thiol-4-amino-5-methylpyrimidine without attempting the isolation of the thioglycolic acid derivative. 2-Thiol-4-amino-5-methylpyrimidine (3.9 gm.) was refluxed with a solution of 4 gm. of chloroacetic acid in 50 ml. of water for 16 hours. Concentrated hydrochloric acid (10 ml.) then was added, and refluxing

was continued for an additional 4 hours. On evaporation to dryness and treatment with ammonium hydroxide, 3.05 gm. (74 per cent) of the basic hydrochloride were obtained. Later experience in the preparation of the 2-carboxymethylthio-4-amino-5-methylpyrimidine (see above) indicated that the time of refluxing with chloroacetic acid was unnecessarily protracted; however, this experiment indicates that there may be some advantage with respect to yield in omission of the isolation of the intermediate.

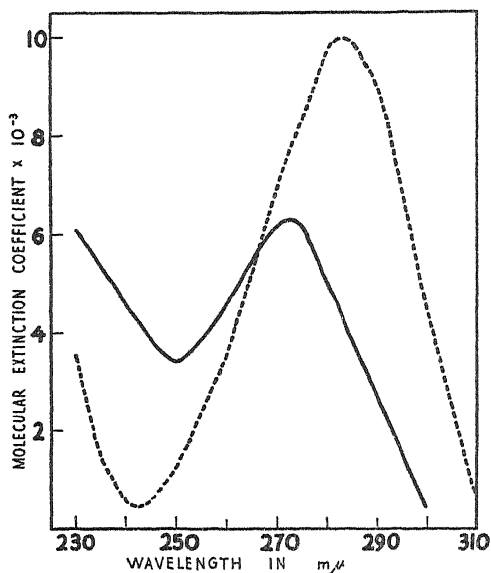


Fig. 1. Ultraviolet absorption spectra of 5-methylcytosine at pH 1, dash line; at pH 11, solid line.

#### SUMMARY

A new and relatively productive method for the synthesis of cytosine and 5-methylcytosine is described. This method is based on the conversion of the requisite 2,4-dithiolpyrimidine to the 2-thiol-4-amino derivative and subsequent hydrolysis of the latter to the 2-hydroxy-4-aminopyrimidine.

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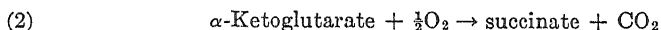
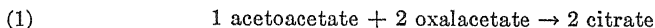
# ANAEROBIC PHOSPHORYLATION DUE TO A COUPLED OXIDATION-REDUCTION BETWEEN $\alpha$ -KETOGLUTARIC ACID AND OXALACETIC ACID\*

BY F. EDMUND HUNTER, JR.

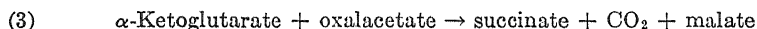
(From the Department of Pharmacology, Washington University School of Medicine, St. Louis)

(Received for publication, July 15, 1948)

During the course of a study (1) of the conversion of acetoacetate to citrate (reaction (1)) it was observed that the oxidation of  $\alpha$ -ketoglutarate to succinate (reaction (2)) had a marked stimulatory effect. In view of



Ochoa's earlier demonstration (2) that reaction (2) results in the formation of several high energy phosphate bonds, it was suggested that the stimulating effect of reaction (2) on reaction (1) might be by way of the creation of these phosphate bonds. The observation that  $\alpha$ -ketoglutarate showed the same stimulating effect on reaction (1) under anaerobic conditions came as a surprise. However, it was soon apparent that this stimulating effect could be reduced to the same basis as under aerobic conditions if one assumed that  $\alpha$ -ketoglutarate was oxidized to succinate according to reac-



tion (3). The oxalacetate had been added in considerable excess to promote quantitative conversion of acetoacetate to citrate. Two pieces of evidence suggested that reaction (3) was actually occurring. (a) The omission of excess oxalacetate in anaerobic experiments deprived  $\alpha$ -ketoglutarate of its stimulatory effect on the removal of acetoacetate. (b) Extra acid was formed when  $\alpha$ -ketoglutarate and oxalacetate were incubated anaerobically with tissue preparations, as expected on the basis of reaction (3). Similar reactions, such as the dismutation of  $\alpha$ -ketoglutarate and the oxidation-reduction between  $\alpha$ -ketoglutarate and acetoacetate have been reported (3-5).

If the aerobic oxidation of  $\alpha$ -ketoglutarate stimulated the conversion of acetoacetate to citrate via creation of energy-rich phosphate bonds, it seemed probable that the anaerobic oxidation of  $\alpha$ -ketoglutarate also generated high energy phosphate bonds, for it had the same stimulatory effect.

\* A preliminary report of this work was presented at the meeting of the American Society of Biological Chemists at Atlantic City, New Jersey, March 15-19, 1948.

Investigation of the anaerobic oxidation of  $\alpha$ -ketoglutarate offered the possibility of gaining information on two points: (a) the mechanism for the stimulatory effect of  $\alpha$ -ketoglutarate on the conversion of acetoacetate to citrate, and (b) the point of origin of one or more of the high energy phosphate bonds formed during  $\alpha$ -ketoglutarate oxidation.

#### EXPERIMENTAL

*Materials*—The sources and methods of preparation were as follows: crystalline sodium pyruvate, a gift of Dr. Ernest Bueding;  $\alpha$ -ketoglutaric acid, part a gift of Dr. L. B. Lockwood, part synthesized (6, 7); acetoacetic acid, Ljunggren (8); oxalacetic acid, Krampitz and Werkman (9); yeast hexokinase, Berger *et al.* (10); adenosine triphosphate (ATP), a modification of the method of Lohmann (11) (the details are almost identical with those recently published by Dounce *et al.* (12)); adenylic acid, from ATP, Kalekar (13); diphosphopyridine nucleotide (DPN),<sup>1</sup> Williamson and Green (14); triphosphopyridine nucleotide (TPN), Warburg *et al.* (15); thiamine pyrophosphate, Merck; flavin-adenine dinucleotide (FAD), gift of Dr. Francis Reithel.

*Methods of Determination*—For inorganic phosphate the methods of Fiske and Subbarow (16) and of Lowry and Lopez (17) have been used. Oxalacetic acid was determined manometrically by decarboxylation (5). Pyruvic acid and  $\alpha$ -ketoglutaric acid were determined by the method of Friedemann and Haugen (18). The  $\alpha$ -ketoglutaric acid values were checked by the enzymatic method of Krebs (5). By this method about 90 per cent of any  $\alpha$ -hydroxyglutaric acid is determined. Comparison of values indicated that only traces of  $\alpha$ -hydroxyglutaric acid were formed. Succinate was determined enzymatically (5). Malic acid was determined manometrically with the malic-oxidizing enzyme from pigeon liver. Directions for preparing this enzyme and advice for use of the method were kindly furnished by Dr. A. Mehler and Dr. S. Ochoa.

*Enzyme Preparations*—The enzyme system studied in this work was first found in the kidney. However, as liver gave somewhat more active preparations, it was used for most of this work. Satisfactory results were found to depend on the exact manner in which the tissue particles were prepared. By the procedure described below it was possible to obtain consistently material with high enzymatic activity. All steps were carried out at low temperature.

The livers from two young adult rats were dropped immediately into distilled water at 0–2° and left there for 4 to 5 minutes with occasional stirring. The livers were then perfused with ice-cold saline to remove the

<sup>1</sup> The author is indebted to Anheuser-Busch, Inc., for considerable amounts of bakers' yeast used as a source for hexokinase and DPN.

blood, and cut up into a stainless steel homogenizer (19). 25 ml. of chilled buffer solution (0.013 M potassium phosphate, pH 7.6, 0.123 M potassium chloride, 0.005 M sodium chloride, 0.0012 M magnesium chloride) were added. The tissue and buffer solution were homogenized at 0–3° for approximately 2 minutes, the exact time for homogenization depending on the speed and fit of the piston. The resultant suspension was diluted to 40 ml. with buffer solution, strained through muslin, and centrifuged in the cold room for 7 to 10 minutes at 8000 R.P.M. The supernatant liquid was discarded and the precipitate washed three times with approximately 30 ml. of buffer solution. Finally the precipitate was suspended in buffer solution to give a volume of 12 to 15 ml.

Other media have been used for homogenization and washing, but the buffer solution high in potassium ions gave the most active preparations. In general three washings of the tissue particles will consistently yield enzyme preparations showing the greatest amount of phosphorylation.

#### RESULTS

*Demonstration That Phosphorylation Occurs under Anaerobic Conditions—*When the tissue preparation was incubated anaerobically without substrate there was a small increase in inorganic phosphate. However, when  $\alpha$ -ketoglutarate and oxalacetate were added, a slight removal of inorganic phosphate was detected. If more than catalytic amounts of adenylyate were provided as phosphate acceptor, the amount of inorganic phosphate disappearing was considerably increased. Experiment 1 in Table I indicates that the inorganic phosphate removed was incorporated into a compound from which it was entirely split by hydrolysis for 10 minutes in 1 N sulfuric acid at 100°, suggesting the conversion of adenylic acid to ATP. While the formation of ATP seemed evident, residual adenosinetriphosphatase activity in the preparation, even in the presence of sodium fluoride, reduced the amount of phosphate remaining in organic combination, making quantitative determination of the phosphate esterification (20) extremely difficult.

When yeast hexokinase was added and glucos<sup>c</sup> substituted for adenylyate as phosphate acceptor, the removal of inorganic phosphate was marked (Tables I and II). The inorganic phosphate which disappeared was incorporated into an organic compound with a resistance to acid hydrolysis corresponding to that for glucose-6-phosphate. Besides being chemically more stable than ATP, this compound probably is less readily hydrolyzed by the tissue enzymes. The extent to which omission of glucose or hexokinase cuts down the phosphorylation is shown in several experiments in Table II. These experiments present a clear cut demonstration of phosphorylation. This phosphorylation was presumably due to reaction (3).

Direct evidence that this reaction occurs will be presented later in this paper.

*Some Factors Essential for Demonstrating Phosphorylation*—To complete the oxidation-reduction enzyme system and achieve optimal phosphorylation, phosphate, magnesium ions, adenylate or ATP, hexokinase, glucose, and perhaps DPN are needed (Table II). These results do not preclude the essentiality of unknown additional factors. Residual adenyl compounds still present in the tissue or formed on the splitting of added DPN (21) could easily account for the low activity without added ATP. The failure of equivalent amounts of adenylate to replace ATP completely may

TABLE I

*Formation of Adenosine Triphosphate and Glucose-6-phosphate*

Each tube contained inorganic phosphate approximately 0.02 M, 0.038 M NaF, 0.006 M MgCl<sub>2</sub>, 0.0012 M ATP, 0.2 mg. of DPN, and enzyme; hexokinase + 50 micromoles of glucose as indicated. Total volume 2.4 ml.; pH 7.6; incubation anaerobically at 30° for 45 minutes. All values are in micromoles. The  $\Delta$  values represent the difference due to  $\alpha$ -ketoglutarate.

Experiment No.	Additions				Inorganic phosphate					
	Oxal- acetate	$\alpha$ -Keto- gluta- rate	Adenyl- ate	Hexo- kinase + glucose	After incu- bation	$\Delta$	After 1 N H <sub>2</sub> SO <sub>4</sub> , 100°			
							10 min.	$\Delta$	180 min.	$\Delta$
1. Dog kidney	100		20		53.5		57.7			
	100	30	20		45.9	-7.6	57.0	-0.7		
	100			+	50.8		51.9			
	100	30		+	42.4	-8.4	46.1	-5.8		
2. Rat liver				+	53.1		53.9		57.4	
		26		+	48.2	-4.9	51.4	-2.5	55.7	-1.7
	100			+	47.1		48.1		57.1	
	100	26		+	35.0	-12.1	38.0	-10.1	45.7	-11.4

be due to the fact that adenosine diphosphate (ADP), more readily available from ATP, is the primary phosphate acceptor.

While DPN did produce some increase in phosphorylation in part of the experiments, it produced no effect in others. It is quite possible that in most cases the tissue particles still contain sufficient DPN for maximal activity. Furthermore, it will be necessary to separate any direct DPN effect from any "ATP-like" effect due to pyrophosphatase type splitting of the DPN molecule (21) with the release of adenylic acid. TPN and FAD did not increase phosphorylation in these preparations.

It was not necessary to add any thiamine pyrophosphate to obtain maximal activity. While evidence indicates that this cofactor is required by



the  $\alpha$ -ketoglutarate-oxidase system, it is released from the protein only by acid treatment (22).

Sodium fluoride was absolutely essential for the demonstration of phosphorylation. The optimal concentration was 0.04 M.

TABLE II  
*Factors Essential for Complete System*

The following were present in all tubes unless otherwise indicated: approximately 0.02 M inorganic phosphate, 0.038 M NaF, 0.006 M  $MgCl_2$ , hexokinase, 0.0012 M ATP, 0.2 mg. of DPN, 55 micromoles of glucose, and rat liver enzyme. Total volume 2.4 ml.; pH 7.6; incubation anaerobically at 30° for 45 minutes. All values are in micromoles.

Experiment No.	Substrates		Omissions or substitutions	Inorganic phosphate			
	Oxalacetate	$\alpha$ -Keto-glutarate		After incubation	$\Delta$ due to $\alpha$ -keto-glutarate	After 10 min. 1 N $H_2SO_4$ , 100°	$\Delta$
1	100			50.0		53.0	
	100	30		35.5	-14.5	37.2	-15.8
	100		Without ATP	46.0*		49.0	
	100	30	" "	38.9	-7.1	39.9	-8.1
	100		" DPN	50.1		50.7	
	100	30	" "	34.1	-16.0	39.6	-11.1
	100		" ATP, DPN	46.5*		47.4	
	100	30	" " "	42.5	-4.0	44.6	-2.8
	100		" glucose	50.5		53.6	
	100	30	" "	44.1	-6.4	50.6	-3.0
2	100			43.0			
	100	30		31.8	-11.2		
	100		Without hexokinase	43.0			
	100	30	" "	36.8	-6.2		
3				61.1		64.0	
		26		58.6	-2.5	64.3	
	100			58.1		63.8	
	100	26		46.3	-11.8	52.5	-11.3
	100		Adenylic for ATP	50.4*		52.9	
	100	26	" " "	44.0	-6.4	48.0	-4.9

\* The inorganic phosphate at the start of incubation was somewhat lower in the tubes without ATP, because the ATP used in the other tubes contained some inorganic phosphate.

*Effect of Aging and Dialysis on Enzyme System*—After 24 hours at 0° the enzyme system showed 40 to 80 per cent of the original activity. The activity lost on aging could not be restored by addition of ATP, DPN, TPN, etc.

Dialysis of the enzyme preparation against more of the buffer solution

used in its preparation resulted in a considerable loss of activity not restored by the combined addition of ATP, DPN, TPN, FAD, thiamine pyrophosphate, etc. In general the dialyzed preparations have not been reactivated by heated tissue extracts. However, such extracts contain numerous substrates and cofactors which greatly complicate the picture by permitting many side reactions to take place.

*Substitution of Other Substrates*—When  $\alpha$ -ketoglutarate is replaced by pyruvate, succinate, citrate, or acetoacetate, little phosphorylation is observed (Table III). In the case of succinate one would not expect oxalacetate to act as an oxidant because of its much lower oxidation-reduction potential. While oxalacetate may act as an oxidant to convert isocitrate to oxalsuccinate, two explanations may be suggested for the failure to obtain phosphorylation: (a) the necessary enzymes may have been washed out of the tissue particles, and (b), even if the reaction occurred, the relatively small difference in potential between the two systems (23) makes coupling with phosphorylation seem unlikely. Pyruvate may show no additional effect because the maximal response has already resulted from pyruvate formed from oxalacetate and is therefore present in the control with oxalacetate alone. Acetoacetate but not pyruvate will replace oxalacetate as an oxidant (Table III).

*Optimal Substrate Concentrations and Incubation Period*—Maximal amounts of phosphorylation are obtained with approximately 0.04 M oxalacetate and 0.015 M  $\alpha$ -ketoglutarate. Doubling the concentration of either substrate results in inhibition. Uptake of inorganic phosphate is very rapid during the first 20 minutes and continues more slowly up to 45 to 50 minutes.

### *Balance Experiments*

Experiments in which a balance was established between the substrates removed and the products formed were carried out with two purposes in mind: (a) to demonstrate directly that the coupled oxidation-reduction reaction was occurring, and (b) to measure the amount of phosphorylation per molecule of  $\alpha$ -ketoglutarate oxidized. The figures (Table IV) for the substances present before incubation all represent values actually determined on aliquots removed at zero time.

*Without Substrate*—During incubation without substrate there is a small increase of inorganic phosphate due to splitting of ATP and other organic phosphates, even with sodium fluoride present (Table IV). When the tissue is incubated with the appropriate substrates, the decrease in inorganic phosphate greatly exceeds the increase mentioned above.

*Oxalacetate Alone As Substrate*—The oxalacetate largely disappears

during incubation (Table IV). No pyruvate accumulates, but roughly two-thirds of the oxalacetate is recovered as malate (any isocitrate formed would have been included with malate by the method of determination used). Insignificant amounts of  $\alpha$ -ketoglutarate are found, but a little succinate does appear.

TABLE III  
*Comparison of Substrates*

All tubes contained inorganic phosphate, 0.038 M NaF, 0.006 M  $MgCl_2$ , 0.0012 M ATP, 0.2 mg. of DPN, hexokinase, 50 micromoles of glucose, and enzyme. Total volume 2.0 to 2.4 ml.; pH 7.4; incubation anaerobically at 30° for 45 minutes. All values are in micromoles.

Experiment No.	Substrates			Inorganic phosphate	
	Oxalacetate	$\alpha$ -Keto-glutarate		After incu- bation	$\Delta$
1. Dog kidney	100			26.6	
		20		25.1	
	100	20		20.0	-6.6
			20 pyruvate	25.2	
	100		20 "	25.8	-0.8
			20 succinate	27.8	
	100		20 "	28.1	+1.5
			20 citrate	28.6	
2. Rat liver	100		20 "	26.4	-0.2
				53.1	
		26		48.2	-4.9
	100			47.1	-6.0
	100	26		35.0	-18.1
			26 pyruvate	50.8	-2.3
3. Rat liver		26	26 "	48.5	-4.6
				42.8	
	80			40.8	-2.0
		30		37.6	-5.2
	80	30		15.0	-27.8
			60 acetoacetate	43.5	+0.7
		30	60 "	22.4	-20.4

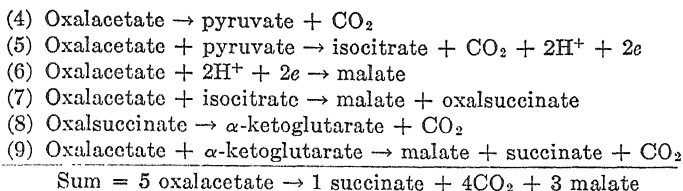
These data are consistent with the suggestion that reactions (4) to (9) account for the removal of the oxalacetate and any pyruvate formed from oxalacetate. The sum of reactions (4) to (9) indicates that 60 per cent of the oxalacetate used would be converted to malate, while the succinate formed would never exceed 20 per cent of the oxalacetate removed. The net result of reactions (4) to (6) would be the formation of 2 malate equivalents for each 3 molecules of oxalacetate removed, thus satisfying this

TABLE IV  
*Balance Experiments*

All flasks contained approximately 0.02 M inorganic phosphate, 0.038 M NaF, 0.006 M MgCl<sub>2</sub>, 0.02 M glucose, 0.0012 M ATP, 0.0002 M DPN, hexokinase, and rat liver enzyme; pH 7.6; incubation anaerobically for 45 minutes at 30°. All values expressed as micromoles per 10 ml. of reaction mixture.

		Before or after incubation	Flask No.	Substrate added	Substance determined					
					Oxalacetate	Pyruvate	Malate	$\alpha$ -Ketoglutarate	Succinate	Inorganic phosphate
Experiment 1										
Before	1	None			3					211
	2	Oxalacetate	323	49	13	5		3.2		218
	3	$\alpha$ -Ketoglutarate	0	4	18	107		8.0		220
	4	Oxalacetate + $\alpha$ -ketoglutarate	350	24	22	101		11.2		218
After	1	None			5					239
	2	Oxalacetate	23	31	205	2		33.6		211
	3	$\alpha$ -Ketoglutarate	0	3	25	89		20		234
	4	Oxalacetate + $\alpha$ -ketoglutarate	44	79	237	3		131		139
$\Delta$ due to reaction in Flask 4*							-77 to -88	+78 to +77		-68 to -72
Experiment 2										
Before	1	None			3					195
	2	Oxalacetate	326	52	2	3		8.5		201
	3	$\alpha$ -Ketoglutarate	0	5	14	106		22		200
	4	Oxalacetate + $\alpha$ -ketoglutarate	365	26	0	120		12.4		203
After	1	None			2					222
	2	Oxalacetate	85	43	194	2		18.7		215
	3	$\alpha$ -Ketoglutarate	0	2	19	86		36		217
	4	Oxalacetate + $\alpha$ -ketoglutarate	87	75	264	7		123		138
$\Delta$ due to reaction in Flask 4*							-92 to -81	+87 to +68		-69 to -77

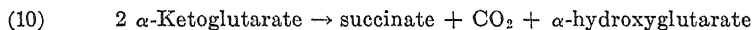
\* The first figure given for  $\Delta$  is the change in Flask 4 during incubation corrected for the changes in Flasks 2 and 3. The second figure is the amount found in Flask 4 after incubation corrected for the amounts found in Flasks 2 and 3. (For phosphate the second figure is calculated from the oxalacetate control alone.)



part of the balance, but no succinate would be formed. The amounts of succinate which appear (Table IV) suggest that 20 to 50 per cent of the substrate undergoes the whole sequence of reactions (4) to (9). Most of these reactions have been studied directly in animal tissues (1, 24-26).

The quantitative data from the balance experiments suggest a correlation between succinate formed and phosphate esterified (30 succinate molecules for 28 phosphate molecules esterified in one case, 10 and 7 in the other). Thus, reaction (9) might account for all of the phosphorylation observed. This would mean that reaction (5), another in which phosphorylation might be expected, apparently did not result in much phosphate uptake in these experiments.

*$\alpha$ -Ketoglutarate Alone As Substrate*—In this case the amount of inorganic phosphate removed is usually very small (Table IV). In each balance experiment 18 to 20 micromoles of  $\alpha$ -ketoglutarate disappeared and 12 to 14 micromoles of succinate appeared. These figures suggest that the major reaction occurring is dismutation of  $\alpha$ -ketoglutarate (5), reaction (10).



This reaction could easily account for the small amount of phosphorylation observed.

*Oxalacetate and  $\alpha$ -Ketoglutarate Together As Substrates*—When both substrates are added, a number of striking changes occur during incubation. (1) The oxalacetate is removed essentially to the same extent as when it is added alone; yet the data indicate a shift to  $\alpha$ -ketoglutarate oxidation as the major reaction. (2) Malate appears in quantities somewhat greater than with oxalacetate alone, and there is a small accumulation of pyruvate. These two substances account for a large proportion of the oxalacetate disappearing. The tendency for a little pyruvate to accumulate suggests that less of reaction (5) and more of reaction (9) takes place. (3)  $\alpha$ -Ketoglutarate, determined by two methods, is almost entirely removed in Flask 4, in contrast to Flask 3. Since by the enzymatic method of determination 85 to 90 per cent of any  $\alpha$ -hydroxyglutarate is included, reduction cannot explain the disappearance of  $\alpha$ -ketoglutarate. (4) Succinate appears in large amounts. After allowance for the changes in the control flasks, it is apparent that  $\alpha$ -ketoglutarate removal and succinate formation are equivalent within the over-all limits of error. This equivalence estab-

lishes directly the oxidation taking place. Since the reduction of oxalacetate to malate is more than sufficient to account for the  $\alpha$ -ketoglutarate oxidation, the balance experiments are direct evidence for the occurrence of a coupled oxidation-reduction. (5) There is a marked uptake of inorganic phosphate, as expected from earlier experiments. The exact value depends on the control value which is subtracted. Since there are two control values, subtracting the sum of the two is the most conservative procedure, and such values are the first ones given. Roughly 1 molecule of phosphate was esterified for each molecule of  $\alpha$ -ketoglutarate oxidized to succinate.

#### DISCUSSION

The failure of earlier workers (26) to demonstrate the coupled oxidation-reduction between  $\alpha$ -ketoglutarate and oxalacetate and to observe the phosphorylation linked with this reaction was undoubtedly due to the use of tissue slices, homogenates, or extracts in which too many side reactions and destructive reactions were occurring. In the present work the use of washed tissue particles permitted a clear cut study of the reaction, because so many complicating reactions were practically eliminated. The addition of hexokinase to transfer phosphate from newly formed ATP to glucose is essential.

In the classical representation of the mechanism for a coupled oxidation-reduction of this type there would be two steps; *viz.*,

- (a)  $\alpha$ -Ketoglutarate + coenzyme  $\rightarrow$  succinate +  $\text{CO}_2$  + reduced coenzyme
- (b) Reduced coenzyme + oxalacetate  $\rightarrow$  coenzyme + malate

The difference between the oxidation-reduction potential of the oxalacetate-malate system ( $E'_0 = -0.100$  to  $-0.180$  volt at pH 7,  $30^\circ$ ) and the oxidation-reduction potential for the  $\alpha$ -ketoglutarate  $\rightarrow$  succinate system ( $E'_0$  calculated to be about  $-0.600$  volt at pH 7.0,  $30^\circ$ ) (23, 27, 28) is more than adequate to account for the creation of one energy-rich phosphate bond in the anaerobic reaction. If there were a high degree of efficiency in the use of the energy, more than one high energy phosphate bond might be formed, for in an oxidation involving 2 electrons a potential difference of about 0.50 volt is equivalent to 24,000 calories.

The identity of the coenzyme or prosthetic group (if one participates in the reaction) remains to be established. The only oxidation-reduction coenzyme which caused any stimulation at all was DPN; yet, as discussed earlier, this relatively small stimulation could easily be explained by effects other than a direct participation. If the reduction of oxalacetate occurs through the regular malic dehydrogenase, DPN is a likely participant, for that enzyme reacts 15 times faster with DPN than with TPN (29). However, TPN is not ruled out.

Since both reactions (a) and (b) of the proposed mechanism involve oxidations, theoretically the uptake of inorganic phosphate and its conversion to high energy phosphate might occur in either step or in both. The exact point of origin of the energy-rich phosphate bond remains to be determined, but the first step seems more probable on the basis of our present knowledge of other oxidative phosphorylations and the energy relationships involved. Preliminary tests with more than catalytic amounts of DPN and reduced DPN have been negative. However, these experiments must be considered critically, because (a) DPN may not be the coenzyme and (b) the preparations used were not 100 per cent pure.

Phosphorylation linked with both steps of the reaction would require very great efficiency in the conversion of energy into phosphate bonds and seems somewhat unlikely. Yet the fact that roughly 1 phosphate molecule is esterified per molecule of  $\alpha$ -ketoglutarate oxidized, even when no corrections are made for phosphatase and adenosinetriphosphatase activity, leads one to speculate about the possibility of two phosphate esterifications. Although sodium fluoride does not completely inhibit the splitting of phosphate from organic combination, the correction to be applied probably would be much smaller than that used by Ochoa (20) because of the addition of generous amounts of hexokinase in these experiments. If 2 orthophosphate molecules were combined to yield 1 inorganic pyrophosphate molecule, by usual criteria it would appear that two phosphorylations had occurred. However, presumably no more than one of these could ever be transferred to glucose, and no evidence has been obtained which suggests pyrophosphate formation.

#### SUMMARY

1. Washed tissue particles from kidney and liver contain an enzyme system which catalyzes an oxidation-reduction between  $\alpha$ -ketoglutarate and oxalacetate under anaerobic conditions.

2.  $\alpha$ -Ketoglutarate is oxidized quantitatively to succinate and carbon dioxide, while oxalacetate is reduced to malate.

3. Addition of purified yeast hexokinase plus glucose makes possible a clear cut demonstration that the above oxidation-reduction is coupled with creation of high energy phosphate bonds.

4. Approximately 1 inorganic phosphate molecule is converted to organic form for each molecule of  $\alpha$ -ketoglutarate oxidized, without applying any corrections for losses in side reactions.

5. Acetoacetate, but not pyruvate, may be substituted for oxalacetate as the oxidant. In general other substances cannot be substituted for  $\alpha$ -ketoglutarate if phosphorylation is to be observed with a well washed tissue preparation.

6. Some properties of the enzyme system are described, and possible mechanisms for the reaction are considered.

The author is greatly indebted to Dr. Carl F. Cori, Dr. O. H. Lowry, and members of their departments for valuable criticism and advice.

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## TISSUE PROTEINS AND CARCINOGENESIS

### II. ELECTROPHORETIC STUDIES ON SERUM PROTEINS DURING CARCINOGENESIS DUE TO AZO DYES

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(Received for publication, August 23, 1948)

It has been known that certain azo dyes will produce cancer of the liver. During the precancerous stages the liver appears to undergo some definite changes. The activity of these azo dye carcinogens is influenced by several dietary factors. Thus the liver can be protected partially from the effects of the dye by simultaneous intake of increased amounts of riboflavin (1). This conforms with the fact that administration of these azo dye carcinogens brings about a decrease in the amount of riboflavin normally present in the liver (2, 3). Feeding of *m'*-methyl-*p*-dimethylaminoazobenzene increases the desoxyribonucleic acid content of the liver (4). Opie (5) and Price *et al.* (6) have observed that a similar dye, *p*-dimethylaminoazobenzene, reduces the ribonucleic acid content of this organ. Miller and Miller (7) have found that *p*-dimethylaminoazobenzene or a metabolite of this compound is bound by a protein constituent of the liver. They found that this azo compound is not bound appreciably by other tissues. However, they observed low levels of bound dye in the proteins of the blood plasma.

In connection with studies on azo dye carcinogenesis, it was considered of interest to determine any changes which might occur in the proteins of the blood serum during the development of liver tumors, hence the investigations reported in this present paper.

#### EXPERIMENTAL

##### *Effects of m'-Methyl-p-dimethylaminoazobenzene on Rat Serum*

*Procedure*—First, electrophoretic experiments were performed on the blood serum from rats fed the active carcinogenic azo dye, *m'*-methyl-*p*-dimethylaminoazobenzene. The animals used were adult male albino rats of the Holtzman Sprague-Dawley strain.<sup>1</sup> They were fed a purified diet (4) which contained an added 0.06 per cent of the *m'*-methyl azo dye. At the end of various periods of time, the blood serum was analyzed for its protein constituents.

<sup>1</sup> Obtained from Holtzman Laboratory Animals, Inc., Madison, Wisconsin.

Samples were taken from the heart upon sacrifice of the animal, groups of three to five rats being used. The samples were allowed to clot and were then centrifuged. The serum was pooled and diluted to 3 times its volume with a sodium diethyl barbiturate buffer<sup>2</sup> of ionic strength 0.1, and a pH of 8.3. The solution so obtained was poured into a Nojax cellulose sausage casing bag<sup>3</sup> and dialyzed against 1.8 liters of the same buffer for 15 or more hours until a Donnan equilibrium was attained.

The electrophoretic experiments were carried out in the Tiselius apparatus<sup>4</sup> with an 11 cm. cell. The cell was filled with the protein and buffer solutions in a cold room at 1–3° and then allowed to equilibrate in a water bath at 0.6°, the temperature at which the electrophoresis was performed. As the serum proteins are all negatively charged at the pH used, the protein boundaries separated and migrated toward the anode. In general, the experiments were continued until the fastest moving albumin component had traveled the length of the cell. The potential gradients used came within the range of 4 to 6 volts per cm.; the lengths of the experiments were 18,000 to 25,200 seconds when the lower potential gradient was used. The initial and final boundary patterns were photographed by the schlieren scanning method of Longworth (9).

*Mobilities*—The electrophoretic mobilities of the proteins of the serum were calculated from the descending pattern by measuring their migration distances from the  $\epsilon$ , buffer-dilution boundary. Conductivity measurements were made on the outer buffer solution used for dialysis.

The mobilities for the components of normal rat serum were calculated. The averages of the values for six experiments, together with the average deviation, are given for each component: albumin  $5.6 \pm 0.1$ ,  $\alpha$ -globulin anomaly<sup>5</sup>  $3.5 \pm 0.2$ ,  $\beta$ -globulin  $2.6 \pm 0.1$ ,  $\gamma$ -globulin  $1.5 \pm 0.1$ , all in units of  $10^{-5}$  cm.  $\times$  (volts per cm.  $\times$  second)<sup>-1</sup>. An  $\alpha$ -globulin anomaly reported by others (10, 11) was generally observed in the descending boundary pattern. Its mobility was calculated in the four out of six cases in which it appeared as a means of identification. The above mobilities appear to be in line with data given by Deutsch and Goodloe (10) and Moore (11) for rat serum at pH 8.6. The values given here are slightly lower, due to the lower pH of 8.3 used.

The mobilities calculated for the components of the many samples of

<sup>2</sup> The sodium diethyl barbiturate buffer of Longworth (8), ionic strength 0.1, and calculated to have a pH of 8.3: 0.050 N NaV-0.020 N HV-0.050 N NaCl, where V = diethyl barbiturate.

<sup>3</sup> Obtained from the Visking Corporation, 6733 West 65th Street, Chicago 38, Illinois.

<sup>4</sup> A modified Klett electrophoresis apparatus.

<sup>5</sup> Average of four values.

serum from rats on the carcinogenic azo dye diets did not differ from those found for the normal serum.

*Composition*—The relative protein composition of each sample was determined. Since the area under each peak is proportional to the total concentration of that component, the percentage of each serum protein present was determined by resolving the pattern into a series of symmetrical curves. The total protein concentration was calculated from Kjeldahl nitrogen analyses. The average value obtained from twenty-nine of the undiluted serum samples was 5.41 gm. of protein per 100 ml. The average deviation was  $\pm 0.52$  gm. per 100 ml.; the range, 4.15 to 6.61 gm. per 100 ml. Two typical electrophoretic patterns are shown. Fig. 1 shows a pattern obtained for serum from normal rats and a characteristic

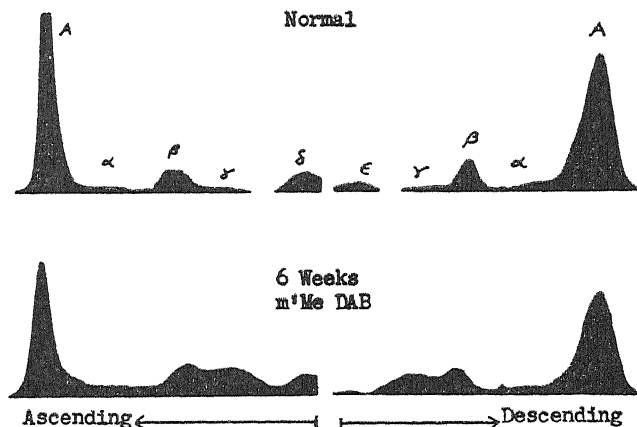


FIG. 1. Electrophoretic patterns of rat sera

diagram for serum from rats in receipt of the *m'*-methyl azo dye. It can be seen that the relative amount of albumin decreased, whereas the percentage of  $\gamma$ -globulin definitely increased in this second case.

The data obtained for the percentage composition of normal serum and serum from rats receiving the *m'*-methyl-*p*-dimethylaminoazobenzene are given in Table I. Since the  $\alpha$ -globulin peaks were ill defined, their areas were estimated together as the total amount of  $\alpha$ -globulin. The average values<sup>6</sup> (and the ranges) for the components of normal rat serum, *viz.* 66 per cent albumin, 14 per cent  $\alpha$ -globulin, 13 per cent  $\beta$ -globulin, and 6 per cent  $\gamma$ -globulin, agree essentially with the values found by Deutsch and Goodloe (10) and Moore (11). In general, three serum samples from

<sup>6</sup> Unless otherwise indicated the serum protein percentages reported in this paper are proportional values and not absolute concentrations.

the rats on the *m'*-methyl azo dye were analyzed after each 2 week interval of feeding. At the end of the first 2 weeks the relative amount of  $\gamma$ -globulin in the serum had definitely increased; the average value was 19 per cent, the range 13 to 27 per cent. The percentage of this component remained high as long as the dye was fed. The values found for the  $\alpha$ - and  $\beta$ -globulin components during the entire 8 week feeding period were normal. The percentage of serum albumin, however, had definitely decreased at the end of 2 weeks to an average value of 53 per cent (44 to 61 per cent). The

TABLE I  
*Effect of m'-Methyl-p-Dimethylaminoazobenzene on Percentage Composition of Rat Sera by Electrophoretic Analysis\**

No. of samples	Diet	Albumin		$\alpha$ -Globulin		$\beta$ -Globulin		$\gamma$ -Globulin	
		Average	Range	Average	Range	Average	Range	Average	Range
		per cent†	per cent	per cent	per cent	per cent	per cent	per cent	per cent
6	Normal	66	60-74	14	11-26	13	10-14	6	2-10
1	1 wk., <i>m'</i> Me-DAB‡	71		11		13		5	
5	2 wks., " "	53	44-61	15	12-16	14	12-17	19	13-27
3	4 " " "	55	54-56	14	13-16	13	11-15	18	16-19
3	6 " " "	58	55-60	14	13-16	13	12-14	16	15-16
3	8 " " "	55	54-58	16	15-17	12	12-14	17	16-18
1	8 " " " + 2 wks., normal (hepatomas)	56		18		16		10	
1	10 wks., <i>m'</i> Me-DAB + 4 wks., normal (hepatomas)	53		25		11		11	
1	2 wks., <i>m'</i> Me-DAB + 1 wk. (normal)	66		17		14		3	

\* Buffer, sodium diethyl barbiturate, ionic strength 0.1, pH 8.3 (8.21-8.40), potential gradient, 4 to 6 volts per cm.

† Percentages are the fractional areas of the electrophoretic diagrams due to each component, and represent the percentages of the total amount of protein in the serum present as these components.

‡ *m'*-Methyl-*p*-dimethylaminoazobenzene, 0.06 per cent in the diet.

relative amount of albumin remained low as long as the dye was continued. Administration of the carcinogenic *m'*-methyl-*p*-dimethylaminoazobenzene over a period of time results in an increased percentage of serum  $\gamma$ -globulin and a concurrent decrease in serum albumin.

Serum from animals to which the dye had been fed for only 1 week was analyzed. The values obtained (Table I) show that the percentage distribution of serum proteins was normal at the end of this period of time.

Two electrophoretic experiments were performed on sera from animals which had developed liver tumors. In the first case, the rats developed

tumors after being fed the diet containing the *m'*-methyl azo dye for 8 weeks followed by a normal diet for 2 weeks. In the second case, the tumors had developed after 10 weeks on the diet containing the *m'*-methyl dye followed by 4 weeks on a normal diet. The percentage of  $\gamma$ -globulin in both of these samples was in the upper part of the normal range, while the percentage of albumin was still somewhat low. The relative amount of  $\alpha$ -globulin appeared to be high in the second sample, although one normal sample had been obtained with an equally high concentration. It is interesting to compare these results with those obtained by Seibert *et al.* (12) from electrophoretic analyses of sera from clinical cases of carcinoma. They found no increase, and often a decrease, in  $\gamma$ -globulin. In cases with metastases to the liver which resulted in jaundice, however, they found an abnormally high  $\gamma$ -globulin. The  $\alpha_2$ -globulin was found to be increased in practically all cases, while the concentration of albumin has been found to be low in carcinoma sera (12, 13).

In an effort to determine whether the drop in the  $\gamma$ -globulin of the sera from the rats with liver tumors could be due to the return to a normal diet, another electrophoretic analysis was made of serum from animals fed the azo dye diet for 2 weeks, followed by a normal diet for the succeeding 3rd week (Table I). In this case, the relative amounts of proteins were normal. Evidently the composition of the blood serum returns rapidly to normal when feeding of the carcinogenic azo dye is discontinued.

#### *Effects of Structurally Related Azo Compounds and One Non-Azo Compound*

Electrophoretic studies were made of sera obtained from rats fed two other azo compounds related structurally to the *m'*-methyl azo dye and one non-azo, but carcinogenic, compound, 2-acetylaminofluorene.

*Azo Compounds*—The serum from rats which had been fed *p*-dimethylaminoazobenzene was studied. This compound is intermediate in carcinogenic activity between the active *m'*-methyl-*p*-dimethylaminoazobenzene and the relatively non-active azobenzene. The tabulated results (Table II) show that the percentage of  $\gamma$ -globulin found in the serum samples after 4 weeks and 8 weeks of feeding was slightly above the normal values. At the end of 3 months of feeding, the serum composition had apparently returned to normal. Administration of an increased amount of *p*-dimethylaminoazobenzene in the diet (0.09 per cent as compared to the usual 0.06 per cent) resulted in approximately 13 per cent of  $\gamma$ -globulin at the end of 2 week and 4 week periods. These amounts were the same as those found with the lower concentration of carcinogen.

The relatively non-carcinogenic compound, azobenzene, produced sera that were essentially normal at the end of 2 and 4 week intervals. The values of 11 and 12 per cent obtained for the  $\gamma$ -globulin were slightly above

normal. It appears from the electrophoresis of sera from rats fed these three structurally related azo compounds that the magnitude of the increase in the percentage of  $\gamma$ -globulin is in line with the relative order of activity of the carcinogenic agents. The most active derivative, *m'*-methyl, definitely produced the most marked effect.

*Non-Azo Compound, 2-Acetylaminofluorene*—Two electrophoretic analyses were made of serum from animals which had been fed the non-azo com-

TABLE II

*Effects of Three Azo Compounds and One Non-Azo Carcinogen on Percentage Composition of Rat Sera*

No. of samples	Diet	Time	Albumin		$\alpha$ -Globulin		$\beta$ -Globulin		$\gamma$ -Globulin	
			Average	Range	Average	Range	Average	Range	Average	Range
		wks.	per cent*	per cent	per cent	per cent	per cent	per cent	per cent	per cent
6	Normal		66	60-74	14	11-26	13	10-14	6	2-10
3	<i>m'</i> Me-DAB†	4	55	54-56	14	13-16	13	11-15	18	16-19
1	DAB‡ (0.06%)	4	61		14		13		13	
1	" (0.06%)	8	59		18		12		12	
2	" (0.06%)	12	66	63-70	16	15-17	12	10-14	6	4- 8
1	" (0.09%)	2	61		14		12		13	
1	" (0.09%)	4	57		17		13		14	
2	Azobenzene	2	59	58-60	17	16-19	13	12-14	11	10-12
2	"	4	61	61-62	16	16-17	11	10-12	11	10-12
1	AAF§	2	63		16		12		8	
1	"	6	62		15		15		8	

\* Percentages are the fractional areas of the electrophoretic diagrams due to each component, and represent the percentages of the total amount of protein in the serum present as these components.

† *m'*-Methyl-*p*-dimethylaminoazobenzene.

‡ *p*-Dimethylaminoazobenzene.

§ 2-Acetylaminofluorene.

pound, 2-acetylaminofluorene. This compound is an active carcinogen; it produces cancer of the liver as well as of other tissues. The analyses showed that at the end of a 2 week and a 6 week interval a normal proportion of 8 per cent of  $\gamma$ -globulin was present. The values for the other serum components were also normal. The absence of any effect of this carcinogen on the blood serum, compared to the increase in  $\gamma$ -globulin caused by the azo dye carcinogens, is noteworthy. However, it may be that longer administration of 2-acetylaminofluorene would produce changes in the composition of the serum.

## DISCUSSION

Administration of the active carcinogenic azo dye, *m'*-methyl-*p*-dimethylaminoazobenzene, in the diet of rats over a period of time resulted in a definite increase in the serum  $\gamma$ -globulin accompanied by a decrease in serum albumin. These results may be considered in the light of other studies related to this work. It has been found, in general, that many pathological conditions result in a reduction of serum albumin and an increase in globulin, usually  $\gamma$ -globulin (13-17). It is also well known that formed antibodies travel with the  $\gamma$ -fraction (18-25). Thus an increased  $\gamma$  component is often associated with antibody formation. Gray and Barron (26) and others (27-29) have shown that liver disease in humans results in a decrease of albumin with a compensatory increase in globulin, usually  $\gamma$ . The magnitude of these changes depends on the degree of liver damage. Therefore, the decreased amount of albumin and the increased  $\gamma$ -globulin content found in the sera of rats fed carcinogenic azo dyes are non-specific changes. However, it appears very interesting that these changes can be correlated with the order of carcinogenic activity of these compounds.

It is also of interest to note the time sequence of liver protein response. The electrophoretic experiments on the blood sera of rats fed *m'*-methyl-*p*-dimethylaminoazobenzene showed marked changes in protein composition at the end of 2 weeks. The first visible signs of liver cirrhosis were observed at 4 weeks. Miller and Miller (7) have found that the binding of the carcinogenic dye, *p*-dimethylaminoazobenzene, first occurs in 4 days. The maximum amount of this dye is found to be bound in the liver at 4 weeks; then the amount of bound dye slowly diminishes to a very small amount at 20 weeks, although the dye is continually being ingested. Since the *m'*-methyl azo dye is a more active carcinogen, it also might be expected to be bound in the liver within 4 days at least.

The protein composition of sera from rats on the carcinogenic non-azo-2-acetylaminofluorene was normal after 2 and 6 weeks. Although the livers of these animals were not completely normal, very little damage could be observed visually.

## SUMMARY

Electrophoretic studies of the relative protein composition of blood serum from rats fed carcinogenic azo dyes led to the following results.

1. Three azo compounds, *m'*-methyl-*p*-dimethylaminoazobenzene, *p*-dimethylaminoazobenzene, and azobenzene, and the non-azo carcinogen, 2-acetylaminofluorene, had no noticeable effect on the mobilities of the serum proteins.

2. Administration of the active carcinogen, *m'*-methyl-*p*-dimethylaminoazobenzene, in the diet resulted in an increased percentage of  $\gamma$ -globulin and a decreased percentage of albumin in the blood serum at the end of 2 weeks. These changes were observed as long as the feeding of the dye was continued. The composition of the serum returned to normal 1 week after the dye was discontinued.

3. Feeding of a non-azo carcinogen, 2-acetylaminofluorene, produced no apparent change in the serum composition after 6 weeks.

4. Comparative studies were made of the effects of three structurally related azo compounds on the composition of the serum. *m'*-Methyl-*p*-dimethylaminoazobenzene is strongly active as a carcinogen, *p*-dimethylaminoazobenzene is intermediate, and azobenzene is relatively non-active. The *m'*-methyl derivative definitely produced the most marked effects on the relative amounts of serum albumin and  $\gamma$ -globulin. The *p*-dimethylaminoazobenzene produced a much smaller effect, while the azobenzene produced little, if any. The magnitude of the changes produced by these azo dyes appears to be in the order of their carcinogenic activity.

It is with pleasure that the authors acknowledge Miss Eleanore Frey's contribution to this research by conducting the series of Kjeldahl nitrogen determinations. We wish to express our appreciation to the American Cancer Society, the Rockefeller Foundation, and the United States Public Health Service for grants which helped to make this work possible.

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THE COMBINATION OF ORGANIC ANIONS WITH  
SERUM ALBUMIN

VI. QUANTITATIVE STUDIES BY EQUILIBRIUM DIALYSIS

A CORRECTION

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(Received for publication, October 18, 1948)

Our attention has been called to an oversight in the calculations in our recent paper.<sup>1</sup> All  $K$  values should be multiplied by 1000/15 and the  $k_1$  values by 15/1000. The  $-\Delta F_1$  values should be corrected by subtracting 2285 calories per mole from the energy values reported.

We are indebted to Dr. Karush of the College of Medicine, New York University, for calling our attention to this error.

<sup>1</sup> Teresi, J. D., and Luck, J. M., *J. Biol. Chem.*, **174**, 653 (1948).



# ESSENTIAL GROUPS FOR THE INTERACTION OF OVOMUCOID (EGG WHITE TRYPSIN INHIBITOR) AND TRYPSIN, AND FOR TRYPTIC ACTIVITY

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(Received for publication, June 29, 1948)

The trypsin inhibitor of egg white has recently been identified as native ovomucoid (1). It has been found also that this trypsin inhibitor has little or no effect on proteolytic enzymes other than trypsin. Thus at least 25 times as much inhibitor was needed to inhibit any of ten other proteolytic enzymes studied as was required to inhibit trypsin.<sup>1</sup> Because of this remarkable specificity, the mode of interaction of trypsin and ovomucoid appears of particular interest. An elucidation of the chemistry of this interaction has been attempted in the present study through the preparation of derivatives of both proteins.

The more important findings include the following: (a) Acetylation of most of the amino groups inactivated neither the inhibitor nor trypsin, but acetylated trypsin was no longer susceptible to inhibition by ovomucoid or its acetyl derivative. (b) In contrast to the amino groups, the carboxyl, phenolic, guanidyl, and possibly other types of groups in ovomucoid were essential for its inhibiting action. (c) The proteolytic activity of trypsin was independent of most of its amino and many of its carboxyl, phenolic, imidazole, and disulfide groups, but it seemed to be dependent upon the integrity of amide, guanidyl, indole, and possibly hydroxyl groups. Thus it appears that *trypsin* combines with the *substrate* through groups other than amino but combines through its amino groups with the acid groups of the *inhibitor*. Other groups in both proteins probably perform important secondary functions in stabilizing the primary ionic combination.

Limited kinetic studies indicate that the inhibition of trypsin by ovomucoid is of the non-competitive type, in agreement with the chemical indications that the substrate and inhibitor combine with different active groups of trypsin.

## EXPERIMENTAL

### *Preparative Methods*

Acetylation was performed by treating the proteins in 5 per cent solution or suspension in half saturated sodium acetate with acetic anhydride (1.2 to

\* Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

<sup>1</sup> Lineweaver, H., and Bean, R. S., unpublished work.

2.4 ml. per gm. of protein distributed over 1 hour), the reaction mixtures being held in an ice bath throughout unless otherwise specified. This technique was previously found to cause acetylation of only the amino groups in a number of proteins (2).<sup>2</sup>

Esterification was achieved by treating 100 mg. of protein with 10 ml. of methanol containing 0.1 N hydrochloric acid (3) for 1 or 24 hours at room temperature or for 4 days at  $-5^{\circ}$ . Methanolic hydrochloric acid appears to be a specific esterifying agent for carboxyl groups in proteins.<sup>3</sup> The esterified preparations and control samples suspended in methanol without HCl were isolated by centrifugation after addition of several volumes of anhydrous ether to precipitate any dissolved protein.<sup>4</sup> They were washed with ether and dried, redissolved in a small volume of water, and lyophilized. The trypsin derivatives were subsequently dialyzed, at least in part, to ascertain the protein nitrogen content of the samples.

An alternative but less effective method of esterification was by means of treatment with 20 per cent propylene oxide in 1 per cent acetic acid (4). Epoxides may combine also with some phenolic and amino groups, even under the most specific, *i.e.* acid reaction conditions (4), but the phenolic groups appeared unaffected in the present experiments.

Partial iodination of the proteins was carried out in 0.6 M acetate buffers, pH 5, by addition of 0.2 ml. of 0.05 N iodine ( $KI_3$ ) solution for 100 mg. of ovomucoid or 140 mg. of trypsin. At  $3^{\circ}$  the solutions remained yellow after 1 to 3 days. The ovomucoid-containing reaction mixture was not completely colorless after an additional day at  $23^{\circ}$ ; the trypsin solution was decolorized after a few hours. For the more intensive iodination of trypsin, 100 mg. of protein were treated with up to 0.6 ml. of 0.1 N iodine in the course of 2 days (final pH 4.8) or for 4 hours at pH 6.3 (phosphate buffer). One experiment was performed in 5 M urea solution at pH 5 (0.5 ml. of 0.1 N iodine, 5 hours).

Coupling of ovomucoid with diazotized sulfanilic acid was performed at room temperature by adding to the protein solutions, buffered at pH 7.6, amounts of *p*-diazobenzenesulfonic acid (in freshly prepared 1 per cent solution in 0.34 M phosphate, pH 7.6), which corresponded to 2.6 or 13 equivalents per  $10^4$  gm. of protein. Trypsin was treated with 1 and 4 equivalents, per  $10^4$  gm. of protein, respectively. The yellow and red reaction mixtures were dialyzed after 30 to 40 minutes. The smaller amounts

<sup>2</sup> Free  $-SH$  groups also react but are absent from most native proteins, including trypsin and ovomucoid.

<sup>3</sup> Two types of groups which do not generally occur in proteins may react; *i.e.*, glycosidic hydroxyl (5) and amido N-methylol groups (unpublished finding of Mr. D. K. Mecham and Dr. H. S. Olcott of this Laboratory).

<sup>4</sup> The activities of both proteins were lowered by no more than 20 per cent by this technique, as compared to isolation by dialysis.

of the reagent probably coupled largely with the imidazole rings of the two proteins, while the larger amounts coupled also with the phenolic rings (6, 2).

Formaldehyde was permitted to act on ovomucoid either for 10 minutes at pH 12 (6 per cent formaldehyde, 2.5 per cent protein), or for 1 and 24 hours at pH 7.6 (2 per cent formaldehyde, 1 per cent protein), or for 4, 2, or 1 day at pH 5, 3, or 2.5 (5 per cent formaldehyde, 7 per cent protein). Trypsin was treated with formaldehyde at pH 3, 5, and 6 (2 or 3 days, room temperature, 4 per cent formaldehyde, 4 per cent protein). In alkaline solution (pH 11 to 12) and in the absence of indole groups (7) only the amide and guanidyl groups of proteins are rapidly and stably substituted (8). At pH 2.5 to 7.6, particularly at high protein concentration (5 to 10 per cent), cross-linking between amide or guanidyl groups, on the one hand, and amino groups on the other occurs at room temperature in the course of 1 to 2 days (8). This same reaction can be rendered more specific and thus more useful by the addition of excess (*e.g.* 5-fold amounts) of simple amides, guanidines, or amines to the protein-formaldehyde reaction mixture. Thus, when much alanine is present, cross-linking occurs preferentially between its amino groups and the amide or guanidyl groups of the protein, the amino groups of the latter remaining largely free. On the other hand, if acetamide or methylguanidine is present in excess, the same reaction leads to the fixation of these compounds to the protein amino groups, its amide or guanidyl groups remaining largely free (8). In a protein containing tryptophan, *e.g.* trypsin, the indole groups also react with formaldehyde at pH 3, but not appreciably at pH 6 (7).

Reduction of ovomucoid and trypsin was achieved by treating 5 per cent solutions at 25° and pH 5.0 (0.15 M acetate buffer) with 5 per cent mercaptoethanol (0.2 ml. per 200 mg. of protein) for time periods ranging from 15 minutes to 24 hours. Ovomucoid was isolated by precipitation and repeated washing with ethanol. It was subsequently redissolved in a small volume of water and again precipitated, washed with ethanol and ether, and dried. Trypsin was isolated either by dialysis against 0.001 M acid, or by precipitation and washing with chilled ethanol, and finally ether.<sup>4</sup> Dialysis led to reoxidation, but after isolation by precipitation, the protein contained 0.5 —SH groups (per 10<sup>4</sup> gm.) as determined with *p*-chloro-mercury benzoate (9). Both proteins were rendered progressively more insoluble with prolonged reducing treatment, probably because of progressive aggregation (2).

In a subsequent experiment, the effect of thioglycol in 1 or 2 M solution at pH 5 was compared with that of the 0.1 or 0.2 M reducing agent at pH 7.6. After 15 to 30 minutes at room temperature, the reaction mixtures were cooled and the products isolated by precipitation and washing with

TABLE I  
*Analytical Characterization of Ovomucoid and Trypsin Derivatives*

Reaction and conditions used to prepare derivatives*	Amounts of various chemical groups in derivatives and controls,† equivalents per 10 <sup>6</sup> gm. protein
Ovomucoid	
Acetylation, 0°, 120 equivalents acetic anhydride	Amino (Van Slyke) 1.0, (ninhydrin) 1.2; acetyl 15.9; Folin (unhydrolyzed) 1.3
“ 0-20°, 120 equivalents acetic anhydride	Amino (ninhydrin) 1.0; acetyl 18.9; Folin (unhydrolyzed) 1.3
Methanol esterification, -5°, 24 hrs.	Methoxyl 2.1
“ “ 23°, 1 hr.	“ 3.5
“ “ 23°, 24 hrs.	“ 6.6
Control, no HCl, 23°, 24 hrs.	“ 0.6
Propylene oxide, 1% HOAc, 3 days	Amino (ninhydrin) 4.0; acid 11.5; Folin (hydrolyzed) 2.8; tyrosine (hydrolyzed) 1.9
Control, 1% HOAc, 3 days	Amino (ninhydrin) 4.8; acid 15.4; Folin (hydrolyzed) 2.6; tyrosine (hydrolyzed) 1.6
Coupling, 2.6 equivalents diazobenzenesulfonic acid, pH 7.6, 30 min.	Tyrosine (hydrolyzed) 1.7; acid 12.9
Coupling, 13 equivalents diazobenzenesulfonic acid	“ “ 1.4; “ 14.7
Formaldehyde, pH 5, 4 days	Amino (Van Slyke) 2.1; acid 11.1; amide 7.0; guanidyl 2.0
“ “ 3, 2 “	Acid 12.0; amide 6.5
“ “ 2.5, 1 day	“ 12.1; “ 6.9
“ + alanine, pH 5, 4 days	Amino (Van Slyke) 3.4; acid 13.5
“ “ “ 3.7, 2 days	Acid 15.2
“ “ “ 3.3, 1 day	“ 14.2
“ + acetamide, pH 5, 4 days	Amino (Van Slyke) 2.0; amide 9.6
“ “ “ 3, 2 “	Amide 9.6
“ “ “ “ 2.6, 1 day	“ 10.8
“ + methylguanidine sulfate, pH 5, 4 days	Amino (Van Slyke) 2.6; amide 6.7; guanidyl 4.4
“ “ “ “ 3.1, 2 days	Guanidyl 3.6
“ “ “ “ 2.7, 1 day	“ 3.0



Deamination, pH 4.2, 15 min.	Amino (ninhydrin) 2.4; tyrosine (hydrolyzed) 1.4
Iodination, 1 equivalent I, pH 5, 30 hrs.	Iodine 0.13; tyrosine (hydrolyzed) 1.5
Sulfation, H <sub>2</sub> SO <sub>4</sub> , -18°, 15 min.	Sulfate 25; Folin (unhydrolyzed) † 1.1
Phosphorylation, P <sub>2</sub> O <sub>5</sub> , 3 days	Phosphate 18; Folin (unhydrolyzed) 1.5
Glucose, 53°, pH 7.6, 3 days	Amino (Van Slyke) 2.3; guanidyl 1.4
" 53°, " 7.6, 14 "	" 1.5; " 0.8
Acetylation, then glucose, 53°, 14 days	Guanidyl 1.2
Untreated ovomucoid	Amino (Van Slyke) 4.3; acid 11.8; acetyl 12.2 (ninhydrin) 3.9
	Folin (hydrolyzed) 3.4; † (unhydrolyzed) 1.45; tyrosine (hydrolyzed) 1.7; ‡ amide 6.8; guanidyl 1.8
Trypsin	
Acetylation, 0°, 120 equivalents acetic anhydride	Amino (Van Slyke) 3.3; acetyl 6.0; basic 4.2
" 0°, 240 "	" 2.0; " 7.2
Methanol esterification, -5°, 3 days	Methoxyl 1.6 (after dialysis 0.7) §
" 23°, 1 hr.	" 1.9
" 23°, 24 hrs.	" 3.3 (after dialysis 2.9) §
Propylene oxide, 1% HOAc, 2 days	Amino (Van Slyke) 10.0; acid 8.5
Control, 1% HOAc, 2 days	Folin (hydrolyzed) 5.8; tyrosine (hydrolyzed) 3.1
Formaldehyde, pH 3.0, 2 days	Acid 8.9
" + alanine, pH 3.7, 2 days	Amino (Van Slyke) 3.6; acid 9.8
" + acetamine, pH 3.0, 2 days	Amide 10.4; guanidine 0.8; tryptophan (1.1)
" + methylguanidine sulfate, pH 3.0, 2 days	Amino (Van Slyke) 4.8; acid 15.2; tryptophan (1.4)
" + acetamide, pH 5.8, 3 days	(Product insoluble); amide 14.0
" + alanine, pH 5.2, 2 days	Amino (Van Slyke) 3.6; guanidyl 3.1; tryptophan (1.2)
" " 5.7, 2 "	" 2.0; amide 17.0; tryptophan 1.8
" " 5.7, 2 "	" 6.5; acid 12.7; " 1.3
Acetylation, then formaldehyde, pH 2.9, 2 days	Amino (Van Slyke) 7.9; " 12.0; tryptophan 1.6
Deamination, pH 4.2, 0°, 30 min.	Tryptophan (0.9)
" 4.2, 23°, 30 "	Amino (Van Slyke) 5.3; basic 6.1
	" 3.9; " 3.7

TABLE I—*Concluded*

Reaction and conditions used to prepare derivatives*	Amounts of various chemical groups in derivatives and controls, † equivalents per 10 <sup>4</sup> gm. protein
<i>Trypsin—continued</i>	
Coupling, 1.0 equivalent diazobenzenesulfonic acid, pH 7.6, 30 min.	Acid 8.6
Coupling, 4.0 equivalents diazobenzenesulfonic acid, pH 7.6, 30 min.	" 9.8
Iodination, 1.3 equivalents I, pH 5, 6 hrs.	Iodine 0.3; Folin (hydrolyzed) 5.3; tyrosine (hydrolyzed) " 2.0; tryptophan 2.0
" 5 " " 5, 2 days	" 2.3
" 6 " " 6.3, 4 hrs.	" 2.7
Acetylation, then iodination, 6 equivalents I, pH 6.3, 4 hrs.	Sulfate 9.3
Sulfation, H <sub>2</sub> SO <sub>4</sub> , -18°, 15 min.	Amino (Van Slyke) 7.9; acid 8.1; basic 8.2
Untreated trypsin	Folin (hydrolyzed) 5.8; tyrosine (hydrolyzed) 3.1; acetyl 1.8; tryptophan 2.0
	Amide 10.7; guanidyl 0.6

\* The amounts of reagent used, when given, are expressed in terms of equivalents per 10<sup>4</sup> gm. of protein. All experiments were performed at room temperature (22-25°), unless otherwise specified.

† In this table, "Folin" stands for apparent phenol, as tyrosine, determined by the Folin method; the various amino acids named stand for the amino acid residues in the proteins as revealed by analyses made either before or after hydrolysis, depending on the case involved. The values were not corrected for moisture.

‡ After hydrolysis with 6 N HCl (18 hours) 1.6 and 1.9 equivalents of tyrosine were found by the Thomas and Folin procedure, as compared to 1.8 and 2.4 equivalents in untreated ovomucoid similarly hydrolyzed. Some sulfonation of the benzene ring thus has occurred (11). The great difference in the apparent tyrosine content of both acid and alkaline hydrolysates of ovomucoid, as determined by the Folin and the Thomas method, is peculiar to this protein and may be due to its high carbohydrate content. The value obtained by the Thomas method is supported by preliminary microbiological assays of Dr. J. C. Lewis of this Laboratory and is now regarded as approximately correct, instead of the value previously published (1).

§ The methoxyl content of esterified trypsin was expressed in terms of the protein present, as ascertained by dialysis. To forestall autolysis, dialysis of trypsin and all its derivatives was done against 0.001 M hydrochloric or sulfuric acid. The lower methoxyl content found after dialysis may thus be due to saponification.

|| The color was not the typical blue, but of purple tint, as previously observed with indole-formaldehyde reaction products (7).

90 per cent acetone-0.01 N hydrochloric acid. They were found to contain 0.3 to 0.7 —SH groups, per  $10^4$  gm. of protein, which were very rapidly autoxidized in aqueous solution.

<sup>4</sup> Reoxidation of the ovomucoid was achieved by aeration of solutions to which a small crystal of copper sulfate had been added, until the nitroprusside test was negative.

*Miscellaneous Reactions*—Partial deamination was obtained by treating 5 per cent solutions of ovomucoid or trypsin in 0.75 M acetate buffer, pH 4.2, with M sodium nitrite at room temperature for 15 minutes (10). The reaction was stopped by the addition of much alanine. A similar experiment performed at 0° for 30 minutes achieved less deamination, but probably caused less reaction of the phenolic residues than the experiment performed at room temperature.

Reactions with glucose were induced by holding solutions containing 8 per cent ovomucoid, or its acetyl derivative, 30 per cent glucose, and 0.5 M phosphate buffer, pH 7.6, at 53° for 3 or 14 days.<sup>5</sup>

Sulfation of the aliphatic hydroxyl groups with concentrated sulfuric acid was performed at -18° according to Reitz *et al.* (11), phosphorylation with phosphorus pentoxide according to Ferrel *et al.* (12).

The ovomucoid derivatives, unless otherwise stated, were isolated by thorough dialysis (2 to 3 days against running tap water at room temperature and 3 days against distilled water at 3-5°), followed by lyophilization. The trypsin derivatives were dialyzed no more than 4 hours against running tap water, then for 8 to 10 days against 0.001 M sulfuric or hydrochloric acid at 3°. Any insoluble protein was removed by centrifugation.

### *Analytical Methods*

The extent of acetylation was ascertained by amino nitrogen analysis (13, 14) and, at times, by approximate determination of the amount of acetyl bound. To this end 30 to 50 mg. of protein were hydrolyzed in a sealed tube with 1 ml. of 6 N  $H_2SO_4$  at 100°. The hydrolysate was washed into a vacuum distillation apparatus and distilled at 50-70° bath temperature and 20 mm. pressure with repeated additions of water. The volatile acid was titrated in the distillate with 0.02 N sodium hydroxide and phenolphthalein. Blank values for untreated proteins ranged from 1 to 3 equivalents (per  $10^4$  gm.) and were not measurably higher if the protein had been treated with acetate buffer as a control to the acetylation procedure.

Methoxyl analyses (15), after humidification (3), served as a measure of the extent of esterification with methanol. Acid group analyses (16) were used as a measure of the extent of esterification with propylene oxide-

<sup>5</sup> A study of the effect of glucose on serum albumin under such conditions is in preparation for press (A. Mohammad *et al.*).

treated samples. The introduction of acid groups into the protein, either through coupling with *p*-diazobenzenesulfonic acid or through formaldehyde and alanine, was also demonstrated by the dye method (16). The introduction of acetamide was determined by analyses for amide N (8) and that of methylguanidine by the Sakaguchi reaction (17). The addition of formaldehyde to the tryptophan residues was shown with the Horn and Jones method (18). The Folin phenol reagent was used, at times without hydrolysis, and at times after alkaline hydrolysis (5 *N* sodium hydroxide, 5 hours at 120°) to ascertain whether the phenolic hydroxyl group was unaffected (19). The Thomas method was applied to tyrosine determinations in alkaline hydrolysates (20).

Sulfate was determined according to Mease (11, 21); phosphate according to Allen (22). Basic groups were determined by the dye method (16). The extent of reduction was ascertained by titration with *p*-chloromercury benzoate (9), with nitroprusside as outside indicator. The reaction proceeded readily at pH 5. The analytical results obtained with ovomucoid and trypsin derivatives are summarized in Table I.

#### *Assay Methods*

Trypsin activities were determined by the method of Anson (23). However, indications were obtained that the standard trypsin-tyrosine curve was not a true expression of the activities of all trypsin derivatives at different dosage levels. Thus the assay results in Table III must be regarded as approximations only.

The activities of trypsin derivatives were also determined by a formol titration method with casein after 35 minutes incubation at 37°. Approximately similar results were obtained by both methods. However, assays of increasing amounts of some trypsin derivatives, *e.g.* acetyltrypsin, gave apparently decreasing activities when read off the standard curve prepared with crystalline trypsin. The observed deviations (in both assay techniques) of trypsin derivatives from the standard curves given with crystalline trypsin may indicate some changes in the specificity of the treated enzyme.

Inhibitor activities were determined by mixing aqueous solutions of suitable amounts of trypsin and inhibitor and allowing the mixture to stand at room temperature and pH  $5 \pm 0.5$  for 10 minutes before adding 1 ml. of the mixture to the hemoglobin. The assay was then completed according to Anson. From the trypsin activity results (color values) the inhibitor activities were obtained by interpolation from a standard curve relating color value and mg. of dry egg white (see (1), "Methods" and foot-note to Table V; and unpublished work of Lineweaver and Bean).

*Materials*—Active ovomucoid was kindly prepared by Mr. C. W. Murray

according to a method described recently (1). Crystalline trypsin (salt-stabilized) and soy bean inhibitors were kindly supplied by Dr. Moses Kunitz of the Rockefeller Institute, and the Lima bean inhibitor by Dr. J. D. Greaves, formerly of this Laboratory. Dialysis of the trypsin against dilute acid permitted the isolation of the protein, although about 30 per cent of the tryptic activity and of the nitrogen was lost thereby. It comprised 30 to 40 per cent of the weight of the original salt-stabilized crystalline preparation and contained 15.0 per cent nitrogen (not corrected for moisture).

#### DISCUSSION

*Ovomucoid Derivatives (Table II)*—The finding that ovomucoid retains almost all its activity after most of its amino groups have been blocked by acetyl groups indicates that these amino groups are not involved in the inhibiting action of ovomucoid on trypsin. This conclusion is supported by the finding that little loss of activity was caused by treatment with nitrous acid, or with glucose for 3 days, or by treatment with formaldehyde in the presence of compounds, such as acetamide or methylguanidine, which prevent the involvement of the amide or guanidyl groups of the protein in the reaction (8).

Esterification with methanol causes inactivation which suggests that the carboxyl groups are essential, although the possibility of denaturation by the acid alcohol can never be completely excluded. The alkali lability of ovomucoid (1) precludes attempts to reverse the esterification by alkaline hydrolysis. The inactivation produced by propylene oxide in dilute acetic acid solution is also indicative of the essentiality of carboxyl groups.

Inactivation of ovomucoid by brief treatment with formaldehyde at pH 12 (88 per cent, as compared to the control exposed to the same pH, which in turn had lost 77 per cent of the original activity) indicates the essentiality of amide or guanidyl groups, both of which react rapidly with formaldehyde at pH 12. This conclusion is strikingly borne out by the marked inactivation caused by formaldehyde at pH 3.3 to 5.0 in the presence of alanine, conditions which also promote reaction with amide and guanidyl groups. Formaldehyde alone at pH 2.5 to 7.6 causes only partial inactivation by cross-linking no more of the essential amide or guanidyl groups than the number of amino groups of the protein permits. The slow inactivation produced by glucose is probably due to its reaction with most of the guanidyl groups. These groups thus appear to be essential. No reagent has been used that reacts with the amide group but not the guanidyl group; hence conclusive evidence is lacking for the essentiality of the amide groups for inhibitory activity.

The fact that a small amount of diazobenzenesulfonic acid causes only

TABLE II  
*Effect of Chemical Modification on Trypsin-Inhibiting Activity of Ovomucoid*

Nature of reaction	Reactive groups		Approximate extent of reaction	Activity recovered†
	Type	Approximate occurrence		
		<i>equivalents per 10<sup>1</sup> gm.*</i>	<i>equivalents per 10<sup>1</sup> gm.*</i>	<i>per cent</i>
Acetylation, 0°	Amino	4.3	3.3	95
“ 0-20°				71
Esterification				
Methanol-HCl, -5°, 24 hrs.	Carboxyl	10	1.5	68
“ 23°, 1 hr.			2.9	44
“ 23°, 24 hrs.			6.0	19
Propylene oxide in 1% HOAc	Mainly carboxyl	10	(3.9)	5
Formaldehyde,‡ pH 12, 10 min.	Amide, guanidyl	7 2	2.1	12
“ “ 7.6, 1 hr.			0.5	82
“ “ 7.6, 24 hrs.	Amino + amide§	4.3, 7	1.4	26
“ “ 5, 4 days	Amino + guanidyl§	4.3, 2	2.7	30
“ “ 3, 2 days			2.5	16
“ “ 2.5, 1 day			1.3	26
“ + added acetamide				
pH 5, 4 days	Amino	4.3	2.7	66
“ 3, 2 days			3.5	59
“ 2.6, 1 day			4.3	89
Formaldehyde + added methyl guanidine sulfate				
pH 5, 4 days	“	4.3	4.4	61
“ 3.1, 2 “			5.0	37
“ 2.7, 1 day			3.7	46
Formaldehyde + added alanine				
pH 5, 4 days	Amide, guanidyl	7 2	5.2 7.0	1 1
“ 3.7, 2 days			7.3	4
“ 3.3, 1 day				
Iodination, pH 5, 30 hrs.	Primarily phenol	1.7	0.1	38
Nitrite, pH 4.2, 30 min.	Primarily amino	4.3	1.6	53
Glucose, pH 7.6, 3 days	Amino, guanidyl	4.3 2	1.8 0.6	69
“ “ 7.6, 14 days	Amino, guanidyl	4.3 2	2.6 1.2	3¶
Sulfation, concentrated H <sub>2</sub> SO <sub>4</sub> , -18°, 20 min.	Hydroxyl	(25)	25	5

TABLE II—*Concluded*

Nature of reaction	Reactive groups		Approximate extent of reaction	Activity recovered†
	Type	Approximate occurrence		
Phosphorylation, $P_2O_5$ in $H_3PO_4$ , 3 days	Hydroxyl	<i>equivalents per 10<sup>4</sup> gm.*</i> (25)	<i>equivalents per 10<sup>4</sup> gm.*</i> 18	<i>per cent</i> 10
Coupling, pH 7.6, 30 min. ( <i>p</i> -diazobenzenesulfonic acid)	Imidazole	1.4**	1	54
2.6 equivalents	"	1.4	3	7
13 equivalents	Phenol	1.7		
Reduction, pH 5 (mercaptoethanol)	Disulfide	2.7		
30 min.			0.05	91
180 "			0.5	75
24 hrs.			1.0	43
Reoxidized (aeration)			0.0	77
" "			0.0	59

\* From data of Table I, unless otherwise stated. The uncertain figures are indicated by parentheses. The values were not corrected for moisture.

† As compared to control solutions exposed to similar conditions of pH and temperature.

‡ The extent of reaction is estimated from amounts of formaldehyde bound. For other criteria (*e.g.* introduction of amides, etc.) see Table I.

§ The plus sign indicates that the reaction is a cross-linking of the protein groups mentioned.

|| When acetyl ovomucoid was treated with formaldehyde at pH 5, in the presence or absence of alanine, the inhibiting activity was 0 and 39 per cent, respectively. Addition of acetamide to ovomucoid-alanine-formaldehyde reaction mixtures after 2 days caused no reactivation; with an inverse order of addition (acetamide and, after 2 days, alanine) 27 per cent of the original activity was recovered.

¶ When acetyl ovomucoid was treated in the same manner, about 13 per cent of the activity of the control solution was recovered.

\*\* Preliminary as yet unpublished microbiological data of Dr. J. C. Lewis of this Laboratory.

slight inactivation suggests that the imidazole groups are not essential; on the other hand, the marked inactivation produced with more extensive "coupling" and particularly the partial inactivation produced by very incomplete iodination suggest that most of the phenolic groups must be free for ovomucoid to exhibit its inhibiting action.

The sulfation and phosphorylation experiments indicate that transformation of the aliphatic hydroxyl to acid sulfate ( $-O-SO_3^-$ ) or phosphate ester groups causes extensive inactivation. This may be due to the blocking of the original hydroxyl groups or to the increase in net charge due to

TABLE III  
Effect of Chemical Modification on Proteolytic Activity of Trypsin

Nature of reaction	Reactive groups		Approximate extent of reaction	Activity recovered†
	Type	Approximate occurrence equivalents per 10 <sup>4</sup> gm.*		
Acetylation, 120 equivalents " 240 "	Amino	7.9	equivalents per 10 <sup>4</sup> gm.* 4.5 6	per cent 85 80
Esterification	Carboxyl	5	0.9 1.2 2.6 0.4	94 93 5 8
Methanol-HCl, -5°, 3 days " 23°, 1 hr. " 23°, 24 hrs.	Primarily carboxyl Indole	2	>0.9 4.3§	1
Propylene oxide (in 0.17 M acetic acid)	Amino + amide† " + guanidyl†	7.9, 10.7 7.9, 0.6		10
Formaldehyde (in 0.5 M acetic acid)	Indole, amino "	2, 7.9 2, 7.9	>1, 3.3§ >0.8, 4.3§	9
+ added acetamide + " methyl guanidine sulfate + " alanine	" amide, guanidyl Amino	2, 10.7, 0.6 7.9	>0.6, 7.2   5.9§	0
Formaldehyde + acetamide, pH 5.8¶ " + alanine, pH 5.2; pH 5.7¶	Amide, guanidyl, Indole	10.7, 0.6 2	4.6, 4.0   0.7	67¶ 3, 8
Acetylation, then formaldehyde (in 0.5 M acetic acid)	Indole, amino	2, 2.5	>1.1	4
+ added acetamide + " methylguanidine sulfate + " alanine	" " " " " amide, guanidyl	2, 2.5 2, 2.5 2, 10.7, 0.6	>1.2, 2.7** >0.8, 0.3 >0.8, 5.0	3 17†† 0
Reduction, ‡ 15 min., pH 5 " 30 " " 5 " 2 hrs., " 5 " 4 " " 5 " 15 min., " 7.6 " 30 " " 7.6	Disulfide	1.1	0.2, reoxidized 0.4 Reoxidized " 0.2 0.2	122, 114 121 100 120 75 126



Coupling, pH 7.6, 30 min., 1 equivalent	Imidazole	1.1§§	0.5	100
" " 7.6, 30 " 4	" phenol	1.1, 3.1	1.7	62
Nitrite, pH 4.2, 30 min., 0°	Amino	7.9	2.6	53
" " 4.2, 30 " 23°	" phenol	7.9, 3.1	5.5	3
Iodination, 0.7 equivalents I, pH 5	Phenol	3.1	0.3	100
" " 5.0 " " 5			2.0	75
" " 5.0 " " 5 (5 M urea)				14
" " 6 " " 6.3			2.3	33
Sulfation	Aliphatic OH	21§§	9.3	8

\* See the asterisk foot-note, Table II. The amino N of trypsin is probably lower than it appears from Van Slyke analysis (15 minutes reaction), since the total basic groups were found lower than the sum of amino, imidazole, and guanidyl groups (8.2 *versus* 9.6). Terminal glycine may well be the cause of this discrepancy.

† As compared to control solutions exposed to similar conditions of reaction and dialysis. The nitrogen recovery after dialysis of a control sample that had been held for 2 days at pH 6.5 was only 30 per cent of the original total N; the activity of this material was  $96 \times 10^{-4}$  unit per mg. of protein (as compared to  $200 \times 10^{-4}$  unit per mg. of protein in the undialyzed preparation). From control samples held at pH 4.9 for 2 or 3 days, about 55 per cent of the nitrogen was recovered after dialysis, with  $95 \times 10^{-4}$  tryptic unit per mg. of protein. The nitrogen recoveries of all formaldehyde-treated samples (73 to 90 per cent) were higher than that of the controls or the iodine-treated samples.

‡ The plus sign indicates that the reaction represents a cross-linking between the protein groups mentioned.

§ From amino N determinations.

|| From acid group determinations.

¶ Without acetamide, trypsin is rendered completely insoluble by formaldehyde treatment at pH 6. In the presence of alanine (pH 5.2), 45 per cent of the protein was rendered insoluble.

\*\* From amide N determinations.

†† Product largely insoluble (>70 per cent); only soluble fractions assayed.

‡‡ Reduction, particularly at pH 5, rendered progressively more of the trypsin insoluble (70 per cent of the protein after 30 minutes reaction). At pH 7.6 less insoluble material but progressively more non-protein N was formed (66 per cent after 30 minutes, as compared to 14 per cent in the unreduced control solution, determined with trichloroacetic acid). The above assays represent comparisons on the basis of soluble protein N only. The control trypsin samples lost about 50 per cent of the original activity in the course of the repeated precipitations and washings with ethanol or acetone-HCl.

§§ Preliminary as yet unpublished results of microbiological assays by Dr. J. C. Lewis of this Laboratory.

the introduction of numerous strongly acid groups. However, the possibility that inactivation is due to denaturation cannot be excluded. Control samples exposed to 85 per cent sulfuric or phosphoric acid showed partial inactivation.

Reduction of disulfide bonds at pH 5 for various time periods causes progressive inactivation. Attempts to regenerate activity by reoxidation were unsuccessful.

*Trypsin Derivatives (Table III)*—Acetyl trypsin proved to be of similar enzymatic activity as control solutions exposed to the same sodium acetate buffer and dialyzed in the same manner. Thus many amino groups of trypsin appear to be non-essential for enzymatic activity. This conclusion is supported by the finding that formaldehyde in the presence of acetamide at pH 6 causes little inactivation. The deamination experiment at 0° also indicates that at least some of the amino groups are not essential for tryptic activity. The finding that nitrous acid at room temperature causes marked inactivation suggests that the phenolic groups are essential, but the participation of the indole rings in the reaction with nitrous acid has not been excluded. Iodination of the phenolic groups that will react at pH 5 in the native protein caused little inactivation, but when the reaction was performed in phosphate buffer at pH 6.3 or at pH 5 in the presence of 5 M urea, the iodine was used up much more rapidly and appreciable inactivation resulted. Bowman (24) had previously studied the effect of iodination on both the proteolytic and hypotensive activities of trypsin and found the enzymatic activity to be largely unimpaired, while most of the hypotensive effect upon intravenous injection had been abolished.<sup>6</sup>

The inactivation produced by formaldehyde at pH 3, regardless of the presence of nitrogenous compounds, is indicative of the essentiality of the indole groups which were found to have an altered and lessened chromogenic activity in such derivatives. At pH 5 to 6, however, much more activity was lost during formaldehyde treatment in the presence of alanine than in the presence of acetamide, while the indole groups appeared largely unchanged; these findings suggested that the amide or guanidyl groups were also essential, while the amino groups were not. This latter fact confirms the results of the acetylation experiments described previously. Attempts to regenerate activity from the alanine-formaldehyde derivative by attempting a reversal of the reaction by exposing the product to dimedon in dilute acid were not clearly successful. The activities were unchanged, while control solutions lost 56 and 33 per cent of their activity when the dimedon treatment was performed at pH 4.6 or pH 2, respectively.

<sup>6</sup> Bowman recently found both the proteolytic and the hypotensive activities of trypsin to be lowered by acetylation with ketene. This effect was in part reversible by treatment with alkali and therefore probably due to acetylation of phenolic groups (25).

Esterification of part of the carboxyl groups of trypsin results in no inactivation. But after a 24 hour reaction period, much more methoxyl is introduced and most of the activity destroyed. The marked inactivation produced by propylene oxide is not understood on the basis of its known reactivity. Sulfation of the aliphatic hydroxyl groups causes inactivation. The retention of most of the tryptic activity after coupling of the protein with 1 equivalent of diazobenzenesulfonic acid suggests that the imidazole groups are not essential, since these generally appear to react more readily than do the phenolic groups (2). The greater amount of the reagent used in the second experiment (4 equivalents) must have combined with some phenolic groups, thus supporting the results of the iodination experiments concerning the non-essentiality of the most reactive phenolic groups.

The finding that acetylation causes no inactivation of trypsin is similar to Herriott and Northrop's finding that the amino groups of pepsin are not essential for its enzymatic activity (26, 19). With trypsin, however, this is much more surprising, in view of the greater number of amino groups present in trypsin (about eight per  $10^4$  gm.) than in pepsin (about one per  $10^4$  gm.). Acetyl trypsin appears to differ from the original enzyme in showing greater resistance to autolysis at neutrality. Thus, after exposure to 0.5 M phosphate buffer, pH 7.6, for 20 hours at  $23^\circ$ , acetyltrypsin showed 41 per cent and trypsin 11 per cent of the original activity. Acetyltrypsin is partly (about 20 per cent of the protein) insoluble in 0.001 M sulfuric acid, and more insoluble material separates from the opalescent solutions during storage at  $3^\circ$ . Lyophilization renders the derived protein more insoluble. Because of the protection against autolysis afforded by acetylation, the yield of soluble acetyl trypsin after isolation by dialysis is actually slightly greater (40 per cent) than the amount of unmodified trypsin that is recovered after dialysis. It is of interest to note that recent, as yet unpublished, experiments of Dr. A. K. Balls and coworkers have shown acetyl trypsin to retain the esterase activity (27) of the original protein.

The high enzymatic activity of the reduced as well as of the reoxidized protein appears surprising, particularly in view of the finding of Grob (28) and Peters and Wakelin (29) that tryptic activity was lowered by reducing agents.<sup>7</sup> Reduction in our experiments was effected at pH 5, whereas the other authors used a slightly alkaline pH. When two preparations reduced to approximately the same extent at both pH values were compared, the one prepared at pH 5 appeared more active than the one prepared at pH 7.6, although the instability of trypsin at pH 7.6 and the apparently changed specificities of the reduced enzyme (see "Assay methods") invalidated exact quantitative interpretation of the data. The re-

<sup>7</sup> Because of the possible interference of the reduced protein in the hemoglobin test, the assay was repeated with casein and formol titration. The reduced protein was found similarly active by both methods.

duced, in contrast to the acetylated, protein appeared to be more susceptible to autolysis than the original enzyme, even at pH 5, as indicated by the appearance of non-protein N during the reaction at both pH values.

An interesting fact emerged when the susceptibility of trypsin derivatives to inhibition by ovomucoid and its derivatives was studied (Table IV). Acetyl trypsin was found to resist inhibition by both ovomucoid and its acetyl derivative under the usual test conditions. Thus one property of the protein (enzymatic activity) was resistant to, while another (specific combination with ovomucoid) had been greatly altered or abolished by acetylation. It would thus appear that the amino groups of trypsin are not involved in enzyme-substrate combination but are necessary for enzyme-

TABLE IV  
*Antitryptic Activity of Ovomucoid and Its Derivatives against Trypsin Derivatives*

Enzyme (trypsin derivatives)	Inhibitor	Apparent inhibitor activity	Inhibitor activity compared with activity against original trypsin
		units per mg.*	per cent
Acetyltrypsin	Ovomucoid	1.3, 0.6	16, 7
“	Acetyl ovomucoid	0.4, 0.2	5, 4
Trypsin ester (low CH <sub>3</sub> O)	Ovomucoid	7.0	79
“ “	Acetyl ovomucoid	7.0	86
“ “ (medium CH <sub>3</sub> O)	Ovomucoid	6.0	66
“ “	Acetyl ovomucoid	3.0	38
Reduced trypsin	Ovomucoid	6.4	79

\* The standard curve for untreated trypsin and egg white was used to obtain these units. Since the curve for treated trypsin or treated ovomucoid might differ somewhat from the curve for untreated material, the units are designated “apparent.”

inhibitor combination. Because of this, it was of special interest to find kinetic evidence that the inhibition was non-competitive with the substrate. The data in Table V show that the degree of inhibition is independent of the substrate concentration within experimental error. Furthermore, the trypsin activity values calculated for non-competitive inhibition agree with the observed values within experimental error (Table V). The complete failure of the competitive inhibition theory to represent the trypsin-ovomucoid system is illustrated in Table V by the trypsin activity values calculated from the competitive inhibition Equation 6B<sub>1</sub> of Goldstein (30).

The results of the present study in regard to the essentiality of the various types of protein groups are summarized in an oversimplified manner in Table VI. It was hoped that the chemical approach used in this investi-

TABLE V  
Kinetic Evidence for Non-Competitive Nature of Inhibition

Inhibitor concentra- tion	Observed activities		Calculated activities ( $E = 142 \times 10^{-9}$ M in all cases)					
	Substrate concentrations, per cent		Non-competitive*	Competitive inhibition†				
				$K_I = 20 \times 10^{-9}$		$K_I = 60 \times 10^{-9}$		
			Substrate concentrations, per cent					
		0.5	1.5	$K_I = 20 \times 10^{-9}$	0.5	1.5	0.5	1.5
$M \times 10^3$								
0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
86	0.57	0.56	0.50	0.19	0.37	0.23	0.40	
172	0.25	0.24	0.24	0.10	0.22	0.16	0.34	

\* Non-competitive inhibition values were calculated by application of Equation 3B of Goldstein (30). The value of  $K_I$ ,  $20 \times 10^{-9}$  mole per liter, is for practical purposes the same as the previously reported value,  $17 \times 10^{-9}$  mole per liter, (1). The value of total enzyme,  $E$ , has not been varied arbitrarily to obtain a fit, but is the value obtained from the trypsin activity (units) present in 6 ml. of reaction mixture, which was converted to moles per liter by the use of the values, 34,000 for the molecular weight of trypsin (31) and 0.0255 for the ratio of trypsin units to mg. of trypsin (32).

† Competitive inhibition values were calculated from Equation 6B<sub>I</sub> of Goldstein (30). This equation applies to cases in which free enzyme and combined enzyme are of the same order of magnitude and in which the total enzyme concentration is much less than the total substrate concentration, which is the case. The enzyme-substrate dissociation constant,  $K_s$ , was determined and found to be approximately 1 per cent. This constant is used in the calculations of competitive inhibition values but, of course, is not a part of the equation representing non-competitive inhibition. The values of  $K_I$ ,  $20 \times 10^{-9}$  and  $60 \times 10^{-9}$ , are expressed in moles per liter.

TABLE VI  
Essentiality of Reactive Groups of Trypsin and Ovomucoid for Biological Activity

Groups	Trypsin		Ovomucoid
	Enzymatic action	Inhibitability	Trypsin-inhibiting action
Amino	Not essential	Essential	Not essential
Carboxyl	Partly essential*	Partly essential	Essential
Phenol	" "		"
Disulfide	Not essential*	Not essential	Partly essential
Amide or guanidyl	Essential		Essential
Indole	"		Absent
Imidazole	Probably not essential		Probably not essential
Aliphatic hydroxyl	Probably essential		Probably essential
Sulfhydryl	Absent		Absent

\* The more readily reacting groups were not essential.

gation might reveal a common mechanism for the interaction of trypsin and the various trypsin inhibitors that have been described, even though these substances differ markedly in their physicochemical properties and in their specificities. However, a few preliminary experiments with the inhibitors from soy (33) and Lima beans<sup>8</sup> indicated that different protein groups may be essential for the activity of various inhibitors.

The methoxyl analyses were kindly performed by Miss Rosie Jang, guanidyl group analyses by Dr. J. Pence, most of the other analytical work by Mr. E. D. Ducay. The sulfation and phosphorylation reactions were performed by Mr. R. E. Ferrel. The helpful suggestions and criticisms of Dr. H. S. Olcott are gratefully acknowledged.

#### SUMMARY

1. The trypsin-inhibiting action of ovomucoid is not impaired by various reagents affecting its amino groups, but the intactness of carboxyl, phenolic, guanidyl, and possibly aliphatic hydroxyl and amide groups appears, in varying degree, to be essential for trypsin-inhibiting action. The imidazole groups seem not to be essential. Sulfhydryl and indole groups do not occur in the inhibitor molecule. Reduction of its disulfide bonds causes progressive inactivation.

2. The enzymatic activity of crystalline trypsin was not abolished by extensive acetylation of its amino groups, by iodination of its most reactive phenolic groups, or by esterification of its most reactive carboxyl groups. Neither reduction of disulfide bonds (at pH 5), reoxidation, nor coupling with imidazole groups caused appreciable loss of activity. Groups which seemed essential were indole and amide, and possibly hydroxyl and guanidyl groups. While acetylation of trypsin did not affect its proteolytic activity, the acetylated derivative was almost completely resistant to inhibition by ovomucoid. It appears that the amino groups of trypsin are essential for combination with ovomucoid, but not with the substrate. As predicted from these results, kinetic experiments showed that inhibition of trypsin by ovomucoid was non-competitive with the substrate.

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<sup>8</sup> Prepared according to an unpublished method by Dr. H. L. Fevold and Dr. J. D. Greaves, formerly of this Laboratory.

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## THE COMPOSITION OF THE DESOXYPENTOSE NUCLEIC ACIDS OF THYMUS AND SPLEEN\*

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(Received for publication, July 28, 1948)

While the early workers in this field, such as Miescher and Hoppe-Seyler, appear to have conjectured the macromolecular and complex character of the nucleic acids that they were the first to isolate, this view soon was abandoned in favor of the chemically more attractive tetranucleotide hypothesis, and those students of nucleic acid chemistry (*e.g.* (1, 2)) who still felt that much remained to be discovered worked against the current of their time. In the more recent past, the development of methods for the study and the characterization of high polymers has brought about a revival of interest in the chemistry of macro molecules occurring in nature. As regards the desoxyribonucleic acid of calf thymus, the high molecular character of this extremely asymmetric compound has been demonstrated repeatedly (3-6). That this nucleic acid was considered as the prototype of all desoxypentose nucleic acids is understandable, since it is the only compound of its kind readily available for a detailed chemical investigation, but the implicit assumption on the part of many workers that desoxypentose nucleic acid is a single chemical individual, regardless of the source from which it is obtained, is incorrect. A comparison of the results contained in the present communication with those submitted in an accompanying paper (7) will be of interest in this connection. A recent study from this laboratory (8), as well as a review article (9), has provided a fuller discussion of the pertinent literature. The problem of nucleic acid specificity also has been considered repeatedly (10, 11).

The great part in the activities of the living cell, ascribed at present to the desoxypentose nucleic acids, makes it imperative to perfect a foundation that will make possible the direct consideration of problems of structure, composition, and specificity. Whatever chemical changes are produced in nucleic acids in the course of cellular development or by radiations or mutagens, such as the compounds of the mustard series, will hardly be of the kind that can be revealed by the mere inspection of optical or other physical characteristics.

\* This work has been supported by a research grant from the United States Public Health Service.

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A chemical comparison with respect to identity of the extremely complex compounds of cellular origin, such as nucleic acids, proteins, or polysaccharides, must be based on the nature and the proportions of their constituents, on the sequence in which these constituents are arranged in the molecule, and on the type of linkages which hold them together. Evidently, a decision will be much easier if, as in the proteins, the number of different constituents is very large, quite apart from the important aid rendered by immunochemical procedures. In the case of the nucleic acids, the relatively small number of different components made very attractive the postulation of identity and regularity which, however, has never been demonstrated adequately. It will be understood that analytical deviations from strictly integral, simple proportions, admissible for substances of comparatively small size, become very significant when applying to compounds whose molecular weights range in the millions.

The present study attempts to provide a survey of the distribution of the purines and pyrimidines in hydrolysates of the desoxypentose nucleic acids derived from calf thymus and beef spleen. It is based on the same microprocedures for the separation and estimation of the nitrogenous constituents of nucleic acids (12) that already have served for the investigation of the composition of pentose nucleic acids (8). The paper last mentioned also included a consideration of the hydrolysis methods employed and a critical discussion of the validity of the experimental results. These points, therefore, need not be reviewed here again.

A synopsis of the results with several nucleic acid preparations is later provided in Table V, which lists the molar proportions in which the four nitrogenous constituents were found. In contrast to the pentose nucleic acids (8), cytosine was the base present in the lowest concentration. Although, in respect to composition, the agreement between nucleic acids of thymus and of spleen is surprisingly good, no claim as to identity should be made. It will be seen that the distribution of purines and pyrimidines was far from that required by a tetranucleotide: for 10 molecules of cytosine, there were found about 16 molecules of adenine, 13 of guanine, 15 (or 13) of thymine.

The correlation of the results submitted here with previous findings in the literature is difficult for a number of reasons. Almost all preparations of desoxypentose nucleic acid that were made the object of detailed analytical studies had been isolated with the use of strong alkali and were probably degraded to some extent. Moreover, since "animal nucleic acids" were regarded as of one kind only, the older workers paid no attention to the possible contamination of their material with pentose nucleic acids, and this may, in preparations derived from sources other than thymus and fish sperm, have contributed to serious errors. For the same reason, prepa-

rations of different, and often unstated, origin were frequently analyzed indiscriminately. Two studies are usually adduced in support of the equimolar distribution of the four nitrogenous constituents. Steudel in 1906 (13) reported on the composition of the nucleic acids of thymus and of herring sperm. His percentage figures, though interpreted by him as evidence for the tetranucleotide structure, actually trend toward the values arrived at in the present study; they correspond to the following mole proportions: adenine 2.0, guanine 1.5, cytosine 1.0, thymine 1.7. Levene and Mandel (14), however, reported figures for a nucleic acid from an unnamed source, probably from spleen (15), that seemed to uphold the tetranucleotide hypothesis.

The composition of a highly polymerized preparation of thymus desoxyribonucleic acid appears to have been considered in one instance only, and this by a quite indirect method. Gulland *et al.* (16) interpreted the results of electrometric titrations (17) in conjunction with the determination of the ratio of purine nitrogen to non-purine nitrogen in the hydrolysate (1.6) as signifying that their product contained, for every 4 gm.-atoms of phosphorus, 1.0 mole each of thymine and of guanine, 1.2 moles of cytosine, and 0.8 mole of adenine. These deductions, which are based on a comparison of the titration curves of the nucleic acid itself and of the so called thymic acid to which it gives rise by acid degradation, are not in accord with the findings presented here. But it should be pointed out that the actual results of the titration of Gulland's nucleic acid corresponded to three dissociations attributable to the amino group and to two dissociations of the purine-pyrimidine hydroxyl per 4 P atoms (17, 18), which is in excellent agreement with the molar proportions calculated for Preparation 3 in Table V (3.9 amino groups for 2.6 purine-pyrimidine hydroxyls) and in satisfactory agreement with the other preparations.

The present study concludes with an attempt to characterize the sugar component of the desoxypentose nucleic acid of spleen, hitherto unidentified, by comparing its partition behavior with that of desoxyribose from thymus nucleic acid. The techniques employed have been discussed in a previous paper (8), but because of the instability of the desoxy sugars the hydrolysis was carried out in a different manner. The nucleic acids were in part degraded enzymatically to the nucleoside stage and the sugars liberated by a short treatment with very dilute acid. The two carbohydrates agreed completely in their partition behavior in three different solvents. It may be tentatively concluded that the desoxypentose nucleic acid of spleen contains 2-desoxyribose.

In conclusion, a few words should be said about a matter of more general concern. To what extent constancy of composition applies to macro molecules of biological origin is unknown and can, perhaps, not even be ascer-

tained by existing methods. It is not impossible that many ostensibly homogeneous preparations of cellular origin actually are mixtures of closely related chemical individuals, representative of different positions within the cell. This may be particularly true of the desoxypentose nucleic acids derived from the cell nucleus, if current conceptions of the specific structure of chromosomes and genes are taken into account. Since, in that case, the constant composition of the nucleic acids of the same cell type is merely a statistical expression of the unchanged state of the cell, it becomes a matter of real interest to compare the distribution of constituents in desoxypentose nucleic acids derived from different deficient mutants of the same species.

#### EXPERIMENTAL

##### *Material*

*Desoxyribonucleic Acid of Calf Thymus*—Three highly polymerized preparations were used. The figures for their N and P contents, and the other data which are given in Table I, refer to the dry substances. *Preparation 1* was isolated as the sodium salt from thymus by a method which followed, with a few modifications, that of Hammarsten (1).<sup>1</sup>

*Preparation 2* was a potassium salt derived from the sodium salt, *Preparation 1*, by a purification method that made use of the well known precipitating action of lanthanum salts (1, 19). One experiment only will be described. The lanthanum salt, produced by the addition of 6 cc. of 2 per cent aqueous lanthanum acetate to a solution of 200.8 mg. of the sodium nucleate in 400 cc. of saline, was collected by centrifugation in the cold and washed twice with 0.2 per cent lanthanum acetate. It was then suspended in 50 cc. of M potassium chloride containing 5 per cent potassium oxalate (pH 7.2), and the mixture was shaken mechanically for 43 hours. The bulky conglomerate of fibers, produced by the addition of 150 cc. of absolute alcohol to the viscous milk, was filtered off without delay, washed with 66 per cent and with absolute alcohol, and taken up in 40 cc. of water. The very turbid solution was freed of lanthanum oxalate by centrifugation for 1 hour at 1900 *g*, and the supernatant was dialyzed, filtered through infusorial earth, and again dialyzed for 10 days against many changes of ice-cold distilled water. After one more centrifugation the solution was frozen and evaporated in a vacuum. The *potassium desoxyribonucleate* (*Preparation 2*, Table I) weighed 166.1 mg. (83 per cent of the starting material) and formed a white fiber felt which gave a clear, very viscous solution in water. This potassium nucleate served as the standard in the determinations (20) of the desoxypentose contents of the other preparations which, in terms of this one, varied from 95 to 102 per cent.

<sup>1</sup> This preparation was made in collaboration with Dr. A. Bendich.

*Preparation 3* was isolated by a procedure somewhat similar to that of Gulland *et al.* (16).<sup>2</sup>

*Desoxyribose Nucleic Acid of Beef Spleen*—1800 gm. of cleaned, fresh beef spleen were ground mechanically and washed by suspension in several 2 liter portions of ice-cold physiological saline. The tissue material was removed by pressing through four layers of cheese-cloth, suspended in 1500 cc. of cold M sodium chloride solution (21), and kept overnight in the refrigerator. The insoluble residue was again extracted at 4°, this time with 10 per cent NaCl solution. The combined extracts were centrifuged at 1900*g* for 1 hour and 1.5 volumes of 95 per cent ethanol were added to the supernatant. The sediment, obtained by the centrifugation of the chilled

TABLE I  
*Desoxyribose Nucleic Acids*

Preparation No.*	Source	N	P	Atomic N:P ratio	Ultraviolet absorption				Specific viscosity ( $\eta_{sp.}$ )†		
					Maximum		Minimum		0.2 per cent solution	0.1 per cent solution	0.05 per cent solution
		per cent	per cent		<i>mμ</i>	<i>ε(P)</i>	<i>mμ</i>	<i>ε(P)</i>			
1	Calf thymus	13.4	8.0	3.7					17	5.2	1.8
2	" "	13.7	8.0	3.8	259	6400	232	2700	23	3.8	1.5
3	" "	13.5	7.6	3.9					12	4.2	2.4
4	Beef spleen	14.7	8.8	3.7	259	6500	231	2900	3.3	1.5	0.7
5	" "	14.1	8.6	3.6							

\*Preparations 1, 3, 4, and 5 were sodium salts, Preparation 2 the potassium salt. N was determined by the Dumas method, P by the gravimetric Pregl-Lieb procedure.

† Ostwald-Fenske viscosimeter; distilled water, 30.3°.

mixture, was taken up in 800 cc. of cold 10 per cent NaCl, the solution was clarified at 1900*g*, and the nucleoprotein precipitated by the addition of 1.5 volumes of ethyl alcohol. The stringy precipitate was spooled on a glass hook and thereby separated from the granular material, which was discarded. It then was suspended in physiological saline and deproteinized (22) in the usual manner by nine treatments with chloroform-octanol (8:1). The threads, precipitated by the addition of 2 parts of ethanol, were lifted and the simultaneously produced granular sediment reworked and converted largely to threads by four precipitations from saline with alcohol (*cf.* (23)). The viscous saline solution of the combined threads was diluted sufficiently to permit clarification by centrifuging, and the nucleic acid again precipitated with alcohol. Its saline solution was then dialyzed against running tap water for 60 hours, against ice-cold distilled water for

<sup>2</sup> We are indebted to Dr. S. Zamenhof for help with this preparation.

24 hours, and evaporated in the frozen state in a vacuum, when the *sodium desoxypentose nucleate* was obtained as a white fluff weighing 1.13 gm. (Preparation 4, Table I).

Another sodium desoxypentose nucleate preparation from beef spleen is listed as Preparation 5 in Table I. This fraction was prepared by a combination of the procedures described previously (1, 16, 21).

*Physical Properties*—All nucleic acid preparations formed white fluffs or fibers and yielded viscous solutions in water. Preparations 2, 3, and 4 (Table I) were free from pentose and protein; Preparations 1 and 5 contained traces of the latter. They showed the characteristic absorption spectrum in the ultraviolet with the maxima and minima listed in Table I (distilled water as the solvent). The reasons for the use of the expression  $\epsilon(P)$ , i.e. the atomic extinction coefficient with respect to phosphorus, have been explained previously (23). Viscosity data for some of the preparations likewise are given in Table I. It will be seen that solutions stronger than 0.1 per cent exhibited anomalously high viscosities. The preparations from spleen gave solutions that were less viscous than those of the products from thymus.

#### *Composition of Desoxypentose Nucleic Acids*

*Methods*—The procedures for the liberation, separation, and estimation of the purines and pyrimidines, described in detail with respect to ribonucleic acids in a recent publication (8), were followed exactly.

*Purines and Pyrimidines*—The purines found in the hydrolysates were adenine (absorption maximum at  $262.5\text{ m}\mu$ ) and guanine ( $249\text{ m}\mu$ ); the pyrimidines were cytosine ( $267.5\text{ m}\mu$ ) and thymine ( $264.5\text{ m}\mu$ ). In no case was uracil demonstrated on the chromatograms. The values found for the purine content are arranged in Table II, those for pyrimidines in Table III.

It was pointed out in a preceding paper (8) that the pyrimidine figures that served for the calculation of proportions and composition carried an upward correction of 5 per cent. This was done in order to take into account the retention of a small quantity of pyrimidine compounds in the purine hydrochloride precipitate that was produced by the treatment of the nucleic acid with methanolic HCl, preliminary to the liberation of the pyrimidines by means of concentrated formic acid. The reasons for this procedure have been given (8). With the ribonucleic acids, the extent of the loss in pyrimidines thus incurred could not be estimated easily, since some adenine, carried over into the pyrimidine fraction by the thorough washing of the purine hydrochlorides, would have contaminated the uracil fraction (12). With desoxypentose nucleic acids, however, such estimations could be performed, as adenine and thymine are separated without difficulty.

TABLE II  
*Purine Content of Desoxypentose Nucleic Acids\**

Preparation No.†	Experiment No.	Duration of hydrolysis with N H <sub>2</sub> SO <sub>4</sub>	Adenine	Guanine
		<i>min.</i>	<i>per cent</i>	<i>per cent</i>
1	1	60	10.0	6.4
	2	60	10.7	7.4
2	3	60	9.0	7.9
	4	60	9.2	8.1
3	5	30	9.4	8.8
	6	60	9.0	7.5
	7	60	9.2	8.4
	8	60	9.0	
	9	120	8.6	7.4
	10	180	8.6	7.1
4	11	60	9.4	8.3
	12	60	8.8	8.5
5	13	60	9.7	8.6

\* Each value represents the average of at least six parallel determinations on the same hydrolysate. The solvent system used for the separations was *n*-butanol-diethylene glycol-water (in NH<sub>3</sub> atmosphere) in Experiments 4, 7, 12, and 13; in all others, *n*-butanol-morpholine-diethylene glycol-water was employed (12).

† The preparations are numbered as in Table I. Preparations 1, 2, and 3 were derived from calf thymus, Preparations 4 and 5 from beef spleen.

TABLE III  
*Pyrimidine Content of Desoxypentose Nucleic Acids\**

Preparation No.†	Experiment No.	Cytosine	Thymine
		<i>per cent</i>	<i>per cent</i>
1	1	4.5	8.0
2	2	4.5	7.7
3	3†	4.3	6.8
	4	4.7	7.0
4	5	4.6	8.0
5	6	5.1	8.1

\* Each value represents the average of at least six parallel determinations on the same hydrolysate. *n*-Butanol-water served as the solvent system.

† See Table II for the explanation.

‡ This hydrolysate was also examined for uracil, but none was found. The chromatogram segment removed at the location of uracil yielded an extract that absorbed no ultraviolet light.

When parallel pyrimidine determinations were carried out on two samples of thymus nucleic acid (Preparation 3, Table I), the values obtained by the customary technique (8), which avoided the washing of the purine hydrochlorides, were cytosine 4.3, thymine 6.8 per cent (Experiment 3,

Table III).<sup>3</sup> In the other experiment, the nucleic acid sample was degraded with the aid of methanolic HCl and the purine hydrochlorides were centrifuged and separated from the supernatant in the usual manner.

TABLE IV  
*Desoxypentose Nucleic Acids; Proportions and Balances\**

	Constituent	Preparation 1	Preparation 2	Preparation 3	Preparation 4	Preparation 5
Content in nucleic acid, %	A.	10.0	9.2	9.4	9.4	9.7
	G.	7.4	8.1	8.8	8.5	8.6
	C.	4.7	4.7	4.9	4.8	5.3
	T.	8.4	8.1	7.3	8.4	8.5
Nitrogen in nucleic acid, %	A.	5.2	4.8	4.9	4.9	5.0
	G.	3.4	3.8	4.1	3.9	4.0
	C.	1.8	1.8	1.9	1.8	2.0
	T.	1.9	1.8	1.6	1.9	1.9
N accounted for as % of nucleic acid N	A.	38.7	34.8	36.1	33.1	35.8
	G.	25.6	27.4	30.2	26.8	28.3
	C.	13.3	13.0	13.7	12.4	14.2
	T.	13.9	13.1	12.0	12.7	13.4
	N. A.	91.5	88.3	92.0	85.0	91.7
Purine N						
Pyrimidine N	"	2.3	2.4	2.6	2.4	2.3
Mole per mole P	A.	0.287	0.264	0.284	0.245	0.259
	G.	0.190	0.207	0.237	0.198	0.205
	C.	0.164	0.164	0.180	0.152	0.172
	T.	0.258	0.249	0.236	0.235	0.243
P accounted for as % of nucleic acid P	A.	28.7	26.4	28.4	24.5	25.9
	G.	19.0	20.7	23.7	19.8	20.5
	C.	16.4	16.4	18.0	15.2	17.2
	T.	25.8	24.9	23.6	23.5	24.3
	N. A.	89.9	88.4	93.7	83.0	87.9
Moles per 4 moles P	A.	1.15	1.06	1.14	0.98	1.04
	G.	0.76	0.83	0.95	0.79	0.82
	C.	0.66	0.66	0.72	0.61	0.69
	T.	1.03	1.00	0.94	0.94	0.97

\* The preparations are numbered as in Table I. The following abbreviations are employed in this table: A. = adenine; G. = guanine; C. = cytosine; T. = thymine; N. A. = total nucleic acid.

They were then washed by suspending them in 0.5 cc. of methanol, and gaseous HCl was again passed through the mixture for 3 hours. Following centrifugation, the united supernatants were evaporated and the resi-

<sup>3</sup> The purine hydrochloride mixture isolated in this experiment was subjected to an analysis (cf. foot-note 3 (8)). The values found, *viz.* adenine 9.7, guanine 8.6 per cent, were in good agreement with the data assembled in Table II.



due was hydrolyzed with formic acid as described previously (8). The chromatogram now exhibited four spots, belonging to cytosine and thymine and to some of the adenine (34 per cent of the total) and guanine (20.5 per cent of the total) that had been transferred to the pyrimidine fraction by the washing process. The values found were cytosine 5.2, thymine 7.2 per cent. Since thymine is the pyrimidine best separated from mixtures contaminated with purines (12), it served for the computation of the relative pyrimidine deficit which, in this particular experiment, was 5.9 per cent.

*Proportions and Balances*—The results obtained with the several preparations are compared in Tables IV and V. With respect to the expressions used, reference may be made to the study of ribonucleic acids recently

TABLE V  
*Desoxyribose Nucleic Acids; Molar Relationships\**

	Constituent	Preparation 1	Preparation 2	Preparation 3	Preparation 4	Preparation 5
Molar proportions	Adenine	1.7	1.6	1.6	1.6	1.5
	Guanine	1.2	1.3	1.3	1.3	1.2
	Cytosine	1.0	1.0	1.0	1.0	1.0
	Thymine	1.6	1.5	1.3	1.5	1.4
Average No. of gm.-atoms N per mole constituent		3.8	3.8	3.9	3.8	3.8
Atomic N:P ratio in nucleic acid		3.7	3.8	3.9	3.7	3.6

\* The preparations are numbered as in Table I.

published from this laboratory (8). Preparations 2, 3, and 4 are considered slightly more reliable than Preparations 1 and 5.

*Rate of Purine Liberation*—The rate of cleavage of the purine nucleotides of thymus nucleic acid by heating with N sulfuric acid was followed with Preparation 3 (Table I). The results assembled as Experiments 5 to 10 in Table II are indicative of the relative ease with which the purines are set free; maximal values for adenine and guanine were obtained after 30 minutes. Prolonged heating brought about a certain amount of destruction (Experiments 9 and 10).<sup>4</sup>

<sup>4</sup> One gains the impression that different nucleic acids differ in their readiness to split off purines on treatment with methanolic HCl, which is the first step in the determination of pyrimidines (8). Whereas with the ribonucleic acids and with the desoxyribonucleic acid of thymus 15 to 30 minutes were sufficient to bring about the appearance of purine hydrochlorides, the corresponding preparations from spleen and from tubercle bacilli (7) were much more sluggish, requiring about 2.5 hours.

As a further check, the purine nitrogen was determined in one preparation by precipitation with silver sulfate (24), the experimental procedure of Gulland *et al.* (16) being followed. The purine nitrogen, thus estimated in the sodium nucleate, Preparation 3, Table I, following hydrolysis with  $N H_2SO_4$  for 1 hour at 115–125°, was found as 8.7 per cent, in good agreement with the value of 9.0 per cent recorded in Table IV.<sup>5</sup>

### *Sugar Component of Desoxypentose Nucleic Acids*

The procedure for the identification by chromatography of the sugar component of the purine nucleotides following acid hydrolysis, as applied to the study of the pentose nucleic acids (8), could not be extended directly to the investigation of the desoxypentoses because of the great lability of these sugars. It was first necessary to convert the nucleic acids into a mixture of desoxypentose nucleosides. Substances of this type have, in the past, served for the isolation of the pure desoxy sugar (25, 26). The enzymatic degradation of the nucleic acids to the nucleoside stage was carried out by a mixture of enzymes consisting of purified desoxyribonuclease of pancreas (27)<sup>6</sup> and of an enzyme preparation, derived from *Aspergillus oryzae*, which is designated as "mylase P" (Wallerstein Laboratories, New York).<sup>7</sup> Preliminary assays with this enzyme mixture showed that, under the conditions of the experiments described below, about 65 per cent of the nucleic acid phosphorus was converted to inorganic phosphate within 11 hours.

10 mg. of each nucleic acid preparation were dissolved in 1.5 cc. of veronal buffer of pH 6.7 (containing 15 micromoles of magnesium sulfate), 0.5 cc. of the enzyme solution (containing a total of 100  $\gamma$  of desoxyribonuclease and 5 mg. of mylase P) was added, and the mixture, protected with 0.01 per cent of ethyl mercurithiosalicylate, incubated for 20 hours at 30°. It was then deproteinized by being shaken with chloroform-octanol and centrifuged. Samples of the solutions were removed in order to test, by chro-

<sup>5</sup> It might be mentioned here that the attempts to characterize all nitrogenous constituents in the same nucleic acid sample, based on the precipitation of the pyrimidine nucleotides as uranium salts (8), were extended to the desoxypentose nucleic acids. However, this procedure yielded low figures for the pyrimidines, in confirmation of similar findings with ribonucleic acids (8). For Preparation 2 (Table I) cytosine 3.2, thymine 6.3 per cent were found; for Preparation 4, cytosine 3.7, thymine 6.1 per cent. In the case of the desoxypentose nucleic acids, a portion of the uranium precipitates was insoluble in 2  $N$  HCl and concentrated formic acid.

<sup>6</sup> We are very grateful to Dr. M. McCarty of the Rockefeller Institute for a specimen of this enzyme.

<sup>7</sup> We should like to thank Mr. Philip P. Gray, Wallerstein Laboratories, New York, for this preparation.

matography, for the presence of free sugars at this stage, but none were found. The remaining solutions were adjusted to pH 1.5 by means of N HCl and heated in boiling water for exactly 12 minutes. The hydrolysates were, in portions of 0.01 and 0.02 cc., neutralized on the filter paper with gaseous  $\text{NH}_3$  and subjected to chromatography in three different solvent systems (28). The development was carried out by means of *m*-phenylenediamine dihydrochloride as described previously (29), and the fluorescent zones that formed were observed in ultraviolet light.

Only one, very fast moving sugar component was observed in all hydrolysates, whose position on the chromatogram was, regardless of the solvent system used, identical for Preparations 1 and 3 from thymus and for Preparation 4 from spleen (Table I). The  $R_F$  values, *i.e.* the proportion of the distances of the starting point from the adsorbate and from the solvent front (30), found (at about 28°) for the sugar component of both the thymus and spleen nucleic acids were as follows: (a) in isobutyric acid (saturated with  $\text{H}_2\text{O}$ ), 0.55; (b) in butanol-pyridine (to the upper layer, resulting from the mixture of 1 volume of pyridine, 1.5 volumes of water, and 3 volumes of *n*-butanol, 1 volume of pyridine was added), 0.60; (c) in *n*-butanol-ethanol-water (4:1:5), 0.45.<sup>8</sup> The  $R_F$  values for D-ribose in these solvents, listed in the same order, were 0.44, 0.49, 0.30; for L-rhamnose 0.48, 0.56, 0.40. The carbohydrate present in the desoxypentose nucleic acid of spleen was, therefore, in all likelihood identical with the sugar of thymus desoxyribonucleic acid, *viz.* 2-desoxyribose.

We are indebted to Mr. W. Saschek and Miss R. Rother for the microanalyses.

#### SUMMARY

The present paper continues the study of the composition of nucleic acids. The distribution of the purines (adenine, guanine) and the pyrimidines (cytosine, thymine) in the hydrolysates of several highly polymerized preparations of the desoxypentose nucleic acids of calf thymus and beef spleen was investigated with the aid of a method recently published for the separation and estimation of these nitrogenous constituents in minute amounts.

The composition of both the thymus and spleen desoxypentose nucleic acids was found closely similar, but it was not in accord with the expect-

<sup>8</sup> When thymus nucleic acid was subjected to the treatment customary for the diphenylamine reaction (20), but with the omission of diphenylamine, no fluorescent band was observed in the chromatogram following the application of *m*-phenylenediamine. Control tests were also performed to ascertain the absence of chromatographically demonstrable sugars from the enzyme mixture employed.

tations derived from the tetranucleotide hypothesis. For 10 molecules of cytosine, 16 molecules of adenine, 13 of guanine, and 15 (or 13) of thymine were found.

The sugar component of spleen desoxypentose nucleic acid, which was liberated from a portion of the nucleosides obtained by enzymatic digestion, closely resembled the carbohydrate similarly released from thymus desoxyribonucleic acid in its chromatographic behavior in three different solvents and was tentatively identified as 2-desoxyribose.

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# ON THE NUCLEOPROTEINS OF AVIAN TUBERCLE BACILLI\*

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(Received for publication, July 28, 1948)

The nucleic acids of tubercle bacilli have formed the subject of several studies, among which may be mentioned those by Ruppel (1), Levene (2), and Johnson and his associates (3-5). The work of Menzel and Heidelberger (6) on the fractionation of the proteins of the tubercle bacillus revealed the presence of several protein fractions, rich in phosphorus and in purines, which appeared to be nucleoproteins. The studies of Seibert *et al.* on tuberculin (7, 8) have included experiments on the separation of nucleic acid, present in the crude preparations, from the biologically active protein. The main portion of the nucleic acid preparations studied appears to have belonged to the desoxypentose type; the presence of pentose nucleic acid does not seem to have been recorded.

In connection with work carried out in this laboratory on bacterial glycogen (9) it was observed that borate buffer extracts of ground avian tubercle bacilli contained, in addition to glycogen, a nucleoprotein fraction giving strong color reactions for desoxypentose. This observation provided an opportunity to study a nucleoprotein obtained from the bacterial cells by a mild extraction process at a low temperature that probably suppressed autolytic reactions. Several other disintegration and extraction methods either were unsuccessful or gave inferior results.

The crude nucleoprotein preparations were slightly yellow; they contained a yellow pigment with a blue-green fluorescence and exhibited an absorption peak at 410 m $\mu$ , in addition to the typical ultraviolet spectrum of nucleic acids (Fig. 1). Further fractionation made use of the fact that the principal nucleoprotein fraction was insoluble around pH 4 and could not be precipitated by half saturation with ammonium sulfate. By this procedure a desoxypentose nucleoprotein which contained 3.2 per cent P, and was only slightly contaminated with pentose nucleic acid, could be prepared. The crude preparations, however, contained a much larger proportion of pentose nucleic acid which was removed in the course of the fractionation. The spectra of a crude and of a purified specimen are compared in Fig. 2.

Not much can be said as yet about the nature of the proteins combined

\* This work has been supported in part by a research grant from the United States Public Health Service.

with the nucleic acids. There is very little likelihood that the crude nucleoprotein fractions contained considerable quantities of a basic protein. Attempts to prepare flavianic acid salts were unsuccessful. Whether the purified desoxypentose nucleoprotein, which amounted to slightly more than one-eighth of the entire preparation, contained a basic protein or a protamine cannot yet be decided.

Our knowledge of the chemistry of nucleoproteins, other than the histone nucleates isolated from various animal cells (10) and the protamine nucleates of fish sperm (11), is as yet very scant. In fact, there exist no generally applicable, safe methods for the isolation of genuine nucleoproteins. Each cellular species will, for the time being, have to be treated as a special case. It should, however, be pointed out that in the isolation methods used in the present study drastic procedures (strong salt solutions, high pH, etc.), which could have produced the cleavage of the conjugated protein, were avoided. It is, for this reason, likely that the isolated nucleoproteins existed as such in the bacterial cell.

Other experiments, *e.g.* on the disintegration of the nucleoproteins, included in the experimental part, require no special comment. The fact that the treatment of the nucleoprotein at a high electrolyte concentration with a lanthanum salt resulted in the precipitation of the intact nucleoprotein is of interest in showing that in this conjugated protein the phosphoric acid groups of the nucleic acid were available for combination with lanthanum.

The composition of the nucleic acid contained in the nucleoprotein fractions of avian tubercle bacilli, described here, is discussed in the following paper (12).

## EXPERIMENTAL

### *Isolation of Nucleoproteins*

*Starting Material*—The cultivation and isolation of the tubercle bacilli of the avian strain were carried out as described previously (9). The synthetic Sauton medium (13) was employed at pH 7.4 and 38°. The organisms, following washing and drying in the frozen state in a vacuum, contained 4.7 to 4.9 per cent N. Their subsequent handling was made easier by a preliminary extraction with ice-cold peroxide-free ether (20 cc. per gm. of organisms) for 1 day, which resulted in the removal of 4 to 5 per cent of bacillary lipides.

*Disintegration of Microorganisms*—Numerous attempts to bring about the complete extraction of water-soluble components by methods in which the use of drastic chemical treatment was avoided were not very successful. Among the procedures tried may be mentioned (1) treatment of the bacilli in 0.1 M borate buffer of pH 8.5 with supersonic vibrations; (2) incubation

in the same buffer with crystalline or with commercial trypsin; (3) grinding of the bacilli, mixed with glass powder, at pH 7.5 between rotating glass cones (14), followed by the extraction of the ground material with diethylene glycol. Procedures (1) and (2) were almost completely ineffective; for instance, by the supersonic treatment only 1.1 per cent of the bacterial N was transferred into solution. Procedure (3) had some effect, but no greater effect than the simpler method finally resorted to; the use of diethylene glycol, moreover, complicated the fractionation of the extracted substances.

*Preparation of Crude Nucleoprotein*—In all following experiments the ether-washed dry tubercle bacilli (mixed with a small amount of the extracting fluid to convert them into a stiff paste) were ground with washed, very fine Pyrex glass powder (diameter  $3\ \mu$ ) in a proportion of 2.5 to 5 parts of glass to 1 part of bacilli.

One typical preparation (Fraction 3, Table I) will be described. A mixture of 25 gm. of bacilli and 100 gm. of glass powder was moistened with borate buffer (pH 8.3) and divided into eight portions, each of which was ground for 30 minutes in a mortar. The ground cells were united, shaken in a refrigerator with 500 cc. of the borate buffer for 2 days, and centrifuged<sup>1</sup> for 30 minutes at 4000 R.P.M. (1900g). The strongly opalescent slightly yellow supernatant was decanted through a filter. The centrifugation residue was washed with 500 cc. of borate buffer which then served for the extraction of a second 25 gm. portion of disintegrated bacilli. In this manner a total of 100 gm. of organisms was processed. The extracts were dialyzed against running water for 48 hours, concentrated by pervaporation to about one-third of the original volume, and again dialyzed against ice-cold distilled water for 72 hours. Ethyl mercurithiosalicylate was added (0.01 percent) and the bacterial glycogen removed by sedimentation at 31,000g (9). The supernatants were once more dialyzed and the crude nucleoprotein fraction was recovered by evaporation of the frozen solution in a vacuum (yield 2.7 gm.). The yields varied for different preparations between 2.4 and 3.4 per cent of the starting material.

*Properties of Crude Nucleoprotein*—The analytical composition of two preparations is summarized in Table I. All specimens formed light yellow fine fluffs with a silky sheen, which could easily be dispersed in water or buffer solutions. They were not precipitated by the gradual dilution with water of their solution in M sodium chloride. They were precipitable by trichloroacetic acid and gave positive biuret, xanthoproteic, Millon, and Hopkins-Cole reactions. The tests for the presence of desoxypentoses

<sup>1</sup> These and most other operations were performed at 4°. The centrifugations were carried out in a refrigerated International centrifuge with multispeed attachment.

TABLE I  
*Composition of Nucleoprotein Fractions*

Fraction No.*	Yield	Nitrogen	Phosphorus	Nucleic acid distribution as per cent of total nucleic acid P	
				Pentose nucleic acid P	Desoxypentose nucleic acid P
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
2	2.4	9.1	0.93	41	59
3	2.7	10.0	0.72	35	65
44	0.4	12.1	3.2	13	87

\* Fractions 2 and 3 represent crude nucleoprotein preparations; Fraction 44 (*cf.* Table III) is a purified specimen.

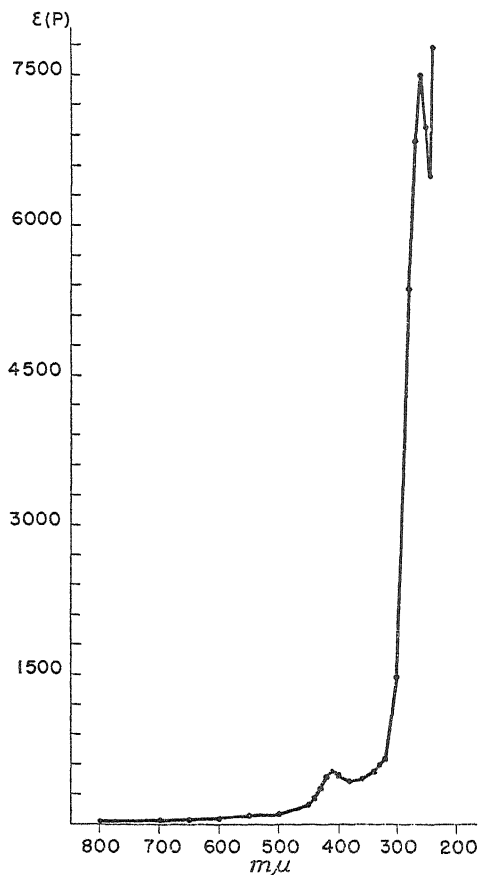


FIG. 1. Absorption spectrum of crude nucleoprotein of avian tubercle bacilli (Fraction 2, Table I) in 0.03 M borate buffer of pH 7.9.



with diphenylamine (15) and with cysteine (16) were likewise positive. The substances gave the Feulgen reaction (17); a small shred of the nucleoprotein, heated with *N* hydrochloric acid for a few seconds, was colored a deep magenta on contact with reduced fuchsin.

Solutions of the crude nucleoprotein fractions were faintly yellow, with a weak blue-green fluorescence which became much stronger in the light of a quartz lamp. The complete spectrum of one preparation is reproduced in Fig. 1. It will be seen that the substance exhibited one weak band in the visible portion of the spectrum, with a center at  $410\text{ m}\mu$  (determined at a concentration of  $18.6\text{ }\gamma$  of nucleoprotein P per cc.) and one very strong band in the ultraviolet at  $259.5\text{ m}\mu$  ( $3.7\text{ }\gamma$  of P per cc.). Absorption minima were observed at  $380$  and  $243\text{ m}\mu$ .<sup>2</sup>

### *Fractionation of Nucleoprotein*

*Inhomogeneity of Crude Nucleoprotein*—After the sedimentation of the bacterial glycogen described before, the nucleoproteins moved as a rule with a single sharp boundary in the electrophoresis cell.<sup>3</sup> Occasionally the extracts contained a small faster component, but in practically all cases they were found to be contaminated with an electrophoretically almost immobile fraction, probably residual glycogen. One of the solutions used showed, for instance, the following components (barbiturate buffer of pH 7.8, ascending mobilities): Component I,  $-0.34$  (24 per cent of the total); Component II,  $-6.9$  (53 per cent); Component III,  $-10.6 \times 10^{-5}$  sq. cm. per volt per second (23 per cent). The scarcity of the available material made attempts at fractionation by electrophoresis impracticable and other ways to reach this goal were chosen.

*Influence of pH on Precipitation of Nucleoprotein*—The experiments presented in Table II demonstrated the existence of a relatively narrow range (pH 3 to 4) within which about 83 per cent of the total N and about 70 per cent of the total P were found in the insoluble portion. At higher pH values the precipitation was negligible.

The nucleoprotein was not precipitated by half saturation with ammonium sulfate at pH 6.3. Under these conditions, the insoluble portion (65 per cent of the total) was found to contain N 13.2, P 0.21 per cent; the supernatant yielded a fraction containing N 7.3, P 1.1 per cent. These findings, *viz.* the flocculation of a nucleoprotein fraction around pH 4, which, however, in contrast to other admixed proteins failed to precipitate in half saturated ammonium sulfate, served for the purification experiments.

<sup>2</sup> The spectroscopic measurements were carried out in the Beckman quartz spectrophotometer. The reasons for the use of  $\epsilon(\text{P})$ , the atomic extinction coefficient with respect to phosphorus, were discussed previously (18).

<sup>3</sup> We wish to thank Dr. D. H. Moore for the electrophoresis experiments.

*Purification of Nucleoprotein*—The dialyzed crude nucleoprotein solution (190 cc.), containing a total of 265.1 mg. of N and 16.1 mg. of P, was brought to pH 4.3 by the addition of 2 per cent acetic acid. The mixture in which a precipitate appeared immediately was chilled for 3 hours and centrifuged. The sediment was washed with ice-cold 0.05 M

TABLE II  
*Influence of pH on Precipitation of Nucleoprotein*

Experiment No.*	pH	Analysis of supernatant				N:P, per cent ratio
		Nitrogen		Phosphorus		
		Weight	Per cent of total	Weight	Per cent of total	
		mg.		mg.		
1	7.2	2.79	100	0.170	100	16.4
2	6.0	2.75	98.6	0.168	98.8	16.4
3	5.1	2.47	88.5	0.156	91.8	15.8
4	4.1	0.50	17.9	0.060	35.3	8.3
5	3.0	0.45	16.1	0.048	28.2	9.4
6	1.8	1.15	41.2	0.056	32.9	20.5

\* Experiment 1 represents the original protein solution in distilled water. The supernatants presented in Experiments 2 to 6 were obtained by the addition of equal amounts of 0.1 M citrate buffers of the indicated pH values and centrifugation, after 20 hours storage at 5°, at 4000 R.P.M.

TABLE III  
*Purification of Nucleoprotein*

Fraction No.	Starting material, fraction No.	Weight	Nitrogen		Phosphorus		N:P, per cent ratio
				Proportion of crude nucleoprotein N (Fraction 4)		Proportion of crude nucleoprotein P (Fraction 4)	
		gm.	per cent	per cent	per cent	per cent	
4				100		100	16.4
41	4	1.68	13.5	85.6	0.73	76.0	18.5
42	4	0.72	4.6	12.5	0.12	5.4	38.3
43	41 (1.67 gm.)	1.36	12.1	62.5	0.20	16.9	60.5
44	41 (1.67 " )	0.29	12.1	13.3	3.2	57.8	3.8

citrate buffer of pH 4.3, dissolved in borate buffer of pH 8.4, and the solution after prolonged dialysis was evaporated in a vacuum in the frozen state. This fraction, listed as Fraction 41 in Table III, formed a slightly yellowish fluff. The supernatants and washings yielded, after dialysis, Fraction 42 (Table III) which, in addition to some protein (almost free of P), contained much glycogen.

The precipitate produced by the addition of 150 cc. of saturated ammonium sulfate solution to 1.67 gm. of Fraction 41, dissolved in 150 cc. of water, was collected by centrifugation, washed repeatedly with half saturated ammonium sulfate, dissolved in borate buffer of pH 8.2, and dialyzed. This solution yielded Fraction 43 (Table III) as a slightly yellow-

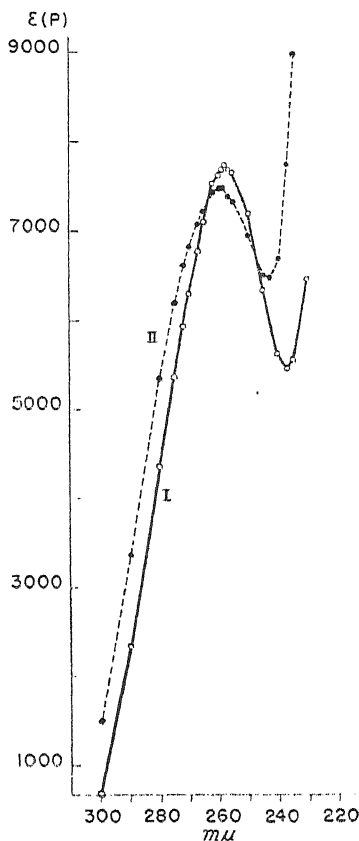


FIG. 2. Ultraviolet absorption of nucleoproteins in 0.03 M borate buffer of pH 7.9. Curve I, purified nucleoprotein (Fraction 44, Tables I and III). Curve II, crude nucleoprotein (Fraction 2, Table I).

ish fine powder. The supernatants from the ammonium sulfate precipitation, freed of electrolytes by prolonged dialysis and evaporated in the frozen state in a vacuum, yielded the purified *nucleoprotein*, Fraction 44 (Table III), which formed a white fiber felt.

As may be gathered from an inspection of Table III, the first purification step, *i.e.* the precipitation at pH 4.3, was accompanied by some loss in

phosphorylated constituents. This probably took place in the course of the dialysis of Fraction 42, as a comparison of the data reported for this fraction in Table III with those for the corresponding preparation in Table II (Experiment 4) will show. The subsequent fractionation with ammonium sulfate, on the other hand, entailed very little loss, 89 and 98 per cent respectively of the N and P contained in Fraction 41 being recovered in Fractions 43 and 44.

*Absorption in Ultraviolet*—Fig. 2 reproduces the absorption spectrum in the ultraviolet of the purified nucleoprotein, Fraction 44 (Curve I). The solution examined at a concentration of 7.0  $\gamma$  of P per cc. had a center of absorption at 258  $m\mu$  and a minimum at 237  $m\mu$ . For purposes of comparison, the detailed spectrum of Fraction 2 (Table I) is likewise given (Curve II). Both nucleoproteins had very similar spectra in the ultraviolet. The fluorescent pigment, however, present in all crude preparations (compare Fig. 1), had disappeared from Fraction 44; it remained for the most part in Fraction 43.

*Nucleic Acid Distribution*—Since the customary colorimetric methods for the estimation of nucleic acids, based on the behavior of the sugar components of the purine nucleotides, cannot be used in the presence of proteins, the approximate distribution of pentose and desoxypentose nucleic acid phosphorus in crude and purified preparations was determined by the method of Schmidt and Thannhauser (19). We are very grateful to Dr. G. Schmidt of Tufts College Medical School for the estimations included in Table I. It will be noted that most of the pentose nucleic acid apparently remained in Fractions 42 and 43 (Table III) and that the purified nucleoprotein, Fraction 44, contained, for the most part, desoxypentose nucleic acid.

*Viscosity*—The viscosity of solutions of the nucleoproteins in 0.1 M borate buffer of pH 7.9 was determined at 37.0° in Ostwald-Fenske pipettes. The values are expressed here as the specific viscosity:  $\eta_{sp.} = \eta/\eta_0 - 1$ , where  $\eta$  and  $\eta_0$  are the viscosities of the solution and the solvent respectively. The substances exhibited a low viscosity. A 0.28 per cent solution of Fraction 2 (Table I) had  $\eta_{sp.} = 0.14$ ; the purified compound Fraction 44 (Tables I and III) had, in a 0.1 per cent solution,  $\eta_{sp.} = 0.28$ .

#### *Disintegration Experiments; Isolation of Nucleic Acids*

*Effect of Proteolytic Enzymes*—The effect of crystalline preparations of trypsin and chymotrypsin on several nucleoprotein samples was studied viscosimetrically at pH 7.9 and 37°. No increase in viscosity was observed. The degradation of the protein moiety by enzymatic action would have been expected to bring about an increase in viscosity owing to the release of nucleic acid (20, 21).

*Disintegration Attempts*—Experiments aiming at the precipitation of the protein moiety as a salt with flavianic acid were unsuccessful, nor could the presence of a basic protein of the protamine or histone types be demonstrated in other ways. Prolonged dialysis against M sodium chloride, a procedure described as productive of splitting in the case of a nucleoprotamine (11), proved ineffective. A solution of 103.6 mg. of Fraction 3 (Table I) in 10 cc. of M sodium chloride was subjected to dialysis against a salt solution of the same concentration at 4° for 8 days. The solution, after being freed of salt, yielded 73.2 mg. of a substance with completely unchanged analytical values; *viz.*, N 9.9, P 0.70 per cent.<sup>4</sup>

*Effect of Lanthanum*—A solution of 370 mg. of the crude nucleoprotein, Fraction 3 (Table I), in 20 cc. of a sodium chloride-borate buffer mixture of pH 7.7 (0.08 M with respect to borate, 1.2 M with respect to NaCl) was clarified by centrifugation at 4800 R.P.M. The gelatinous precipitate pro-

TABLE IV  
*Effect of Lanthanum on Nucleoprotein*

Fraction No.	Properties	Weight	Nitrogen		Phosphorus	
				Proportion of original N		Proportion of original P
		mg.	per cent	per cent	per cent	per cent
31	Soluble at pH 7.7	82.6	4.5	10.0	0	0
32	" " " 4.9	21.3	13.5	7.8	0.19	1.5
33	Insoluble at pH 7.7 and 4.9	157.1	14.1	59.9	1.12	66.1

duced by the addition to the supernatant of 5 cc. of 2.5 per cent aqueous lanthanum nitrate was removed by centrifugation after being chilled overnight, washed repeatedly with the borate-NaCl mixture, and then extracted with 0.1 M acetate buffer of pH 4.9. The insoluble extraction residue, exhaustively washed with distilled water, was suspended in water, and this suspension, as well as the dialyzed supernatants (obtained at pH 7.7 and 4.9), was evaporated in a vacuum in the frozen state. The results, summarized in Table IV, show an interesting parallelism with the fractionation experiments described above. The portion not precipitated by lanthanum (Fraction 31, Table IV) appears quite similar to Fraction 42 in Table III, the major part consisting of material free of P and N, probably glycogen. The entire nucleoprotein, on the other hand, combined with lanthanum and became insoluble at pH 7.7. No indication of a dissociation into nucleic acid and protein in the presence of a high

<sup>4</sup> The small amount of yellow pigment which, as was mentioned above, accompanies the crude nucleoprotein preparations was not removed by the dialysis.

salt concentration was observed. These experiments indicated that the phosphoric acid groups of the nucleic acid were not involved in the combination with protein in such a manner as to make them unavailable for salt formation with lanthanum.

*Action of Saturated Sodium Chloride Solution*—The efficacy of saturated sodium chloride solutions in the cleavage of the nucleic acid-histone bond is well known (22, 23). A solution of 296.5 mg. of Fraction 3 (Table I) in 12 cc. of 10 per cent sodium chloride was saturated with NaCl, after which turbidity and precipitation occurred. The chilled mixture was centrifuged and 2 volumes of alcohol were added to the supernatant. The precipitate thus produced was not all soluble in water and physiological saline. The united insoluble residues were suspended in 0.1 M borate buffer of pH 8.4, subjected to prolonged dialysis, and recovered in the usual manner. The insoluble *protein* fraction weighed 102.7 mg. (35 per cent of the starting material), formed an almost white, light fluff, and contained N 13.8, P 0.4 per cent. The saline solution was freed of residual protein by five treatments for 16 hours each with 9:1 chloroform-octanol (24) and the crude nucleic acid precipitated by the addition of 4 volumes of alcohol. Reprecipitation with 1 volume of alcohol finally removed the yellow pigment accompanying the crude nucleoprotein. The *nucleic acid* preparation obtained in this manner weighed 12.7 mg. and formed white fibers which gave quite viscous solutions. The analytical figures (N 8.2, P 4.8 per cent) showed it to be still contaminated with bacterial glycogen. It could be further purified by the procedures discussed in the following paper (12).

These experiments indicated that the cleavage of the nucleoprotein by saturated salt solutions was not complete: 21 per cent of the original P remained in the precipitated protein fraction, 28 per cent was recovered in the liberated nucleic acid.

*Action of Sodium Desoxycholate*—This very active detergent could be utilized for the splitting of the nucleoprotein (*cf.* (25)). A solution of 820 mg. of Fraction 3 (Table I) in 40 cc. of physiological saline was mixed with an equal volume of 1 per cent aqueous sodium desoxycholate. The mixture (pH 7.6) was chilled overnight and the precipitate produced by the addition of 3 volumes of absolute alcohol removed by centrifugation. It was washed with alcohol and its solution in 30 cc. of saline was shaken with 20 cc. of chloroform-octanol for 20 hours. The coagulated protein portion was recovered, washed with water, 70 and 100 per cent ethyl alcohol, and ether, and dried. It weighed 208 mg. and contained N 14.1, P 0.20 per cent (7 per cent of the original P). The treatment of the saline solution with chloroform-octanol was repeated four times for a total of 90 hours. The nucleic acid was twice precipitated from its solution in saline by the addition of 4 volumes of alcohol and subsequently three times with

1 volume of alcohol. The yellow, blue-fluorescing pigment mentioned before was soluble in 50 per cent alcohol and was thus removed. The nucleic acid, precipitating as a coherent bundle of slimy fibers, was again dissolved in 12 cc. of saline, and the solution was clarified by centrifugation at 11,000*g* for 30 minutes, dialyzed against tap and ice-cold distilled water for a total of 4 days, and evaporated to dryness in the frozen state *in vacuo*. The nucleic acid, 67.2 mg. of white fibers, contained N 10.6, P 6.3 per cent (72 per cent of the original P) and was further purified (12).

Similar nucleic acid preparations could be obtained by the direct extraction of dry ether-washed tubercle bacilli with 0.5 per cent sodium desoxycholate solution, but the yields were smaller and the products more contaminated with polysaccharides.

*Purification of Nucleic Acids*—As pointed out repeatedly, the main obstacle to the preparation of pure undegraded nucleic acids from the bacillary nucleoproteins lay in the difficulty with which contaminating glycogen and other substances were removed. The low percentage values for nitrogen and phosphorus indicated the presence of non-nitrogenous impurities. Purification by electrophoresis proved possible, although it was accompanied by considerable losses. One example will be given here. A crude nucleic acid sample, prepared by extraction of the bacilli with 0.5 per cent sodium desoxycholate, was found to contain P 3.8 per cent (yield 30.7 mg. from 37 gm. of ether-washed bacilli). The entire quantity was dissolved in 2 cc. of barbiturate buffer of pH 7.8 and subjected to electrophoresis in a micro cell. The examination revealed three components with the following descending mobilities: Component I,  $-1.6$ ; Component II,  $-10.9$ ; Component III,  $-15.2 \times 10^{-5}$  sq. cm. per volt per second. Components II and III were removed separately, subjected to dialysis, and recovered by evaporation in the frozen state *in vacuo*. Component II weighed 9.0 mg. and contained P 1.9 per cent (15 per cent of the original P). Component III was nucleic acid; it consisted of white fibers weighing 6.6 mg. and contained N 12.9, P 7.6 per cent (43 per cent of the original P).

#### SUMMARY

The properties and the purification of a nucleoprotein fraction isolated from avian tubercle bacilli (containing desoxypentose and pentose nucleic acids) are discussed. Methods for the cleavage of the nucleoprotein and the isolation of desoxypentose nucleic acid are reported.

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# MICROBIAL NUCLEIC ACIDS: THE DESOXYPENTOSE NUCLEIC ACIDS OF AVIAN TUBERCLE BACILLI AND YEAST\*

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(Received for publication, July 28, 1948)

A preceding paper from this laboratory (1) has shown that the desoxypentose nucleic acids of calf thymus and beef spleen resemble each other very closely in regard to the molar proportions of their constituent purines and pyrimidines. While no claim of identity can be made, since variations in the sequence in which the nucleotide components are interconnected in these two compounds are entirely possible, this similarity of nucleic acids originating in different tissues of the same species is not surprising. However, if, as appears probable (2), certain nucleic acids are endowed with a specific biological activity, a search for chemical differences in nucleic acids derived from taxonomically different species should be conducted, and microorganisms would appear to be one of the most promising sources. The microprocedures developed in this laboratory (3, 4) have made studies of this type possible.

This paper presents, as a first attempt, an investigation of the composition of the desoxypentose nucleic acids derived from avian tubercle bacilli and from yeast.

## EXPERIMENTAL

### *Material*

*Desoxypentose Nucleic Acids of Avian Tubercle Bacilli*—Several nucleic acid preparations obtained by the degradation of the bacillary nucleoprotein fractions, discussed in an accompanying paper (5), were purified via their lanthanum salts by the procedure described previously (1). Two instances will be mentioned here. A sample (40 mg.) of the crude nucleic acid prepared by the action of sodium desoxycholate on the nucleoprotein fraction (No. 3 (5)) (N 10.6, P 6.3 per cent) was converted into the lanthanum salt. This precipitate was shaken with 20 cc. of M potassium chloride (containing 5 per cent potassium oxalate) for 24 hours. (The pH of the mixture was 7.2.) The centrifugation and washing of the sedi-

\* This work has been supported by a research grant from the United States Public Health Service.

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ment with 5 cc. of 0.04 M KCl were followed by precipitation with 60 cc. of absolute alcohol, filtration, and washing with 66 and 100 per cent alcohol and with ether. The fibers obtained by the addition of 10 cc. of alcohol to the solution of the precipitate in 10 cc. of water were dissolved in 30 cc. of water, and the solution, following dialysis for 4 days and centrifugation, was evaporated in the frozen state in a vacuum. The purified *potassium salt of the nucleic acid* (Preparation 1), 21.3 mg. of white fibers, contained N 13.3, P 7.4 per cent (atomic N:P ratio 4.0). The colorimetric estimations were carried out with the potassium salt of thymus nucleic acid (Preparation 2 (1)) as the standard for the reaction with diphenylamine (6) and with yeast ribonucleic acid, purified according to Fletcher *et al.* (7), as the standard for the reaction with orcinol (8). They revealed the presence of about 82 per cent of desoxypentose nucleic acid and of a small amount (8 to 10 per cent) of pentose nucleic acid.<sup>1</sup> Aqueous solutions of the substance were noticeably viscous, but less so than preparations of thymus nucleic acid (1) or of the desoxypentose nucleic acid recently isolated from yeast (9) and discussed later in this paper. A very dilute solution, *viz.* 0.03 per cent in distilled water (30.3°), had  $\eta_{sp.} = 0.2$ . The scarcity of material prevented an examination at higher concentrations. The absorption maximum of this nucleic acid was found at 257 m $\mu$  with an  $\epsilon(P)$  value (9) of 6400. This fraction was used for the study of the distribution of purines and pyrimidines.

Another nucleic acid preparation purified through the lanthanum salt was a substance isolated by the treatment with warm 10 per cent NaCl of the bacterial residues from the nucleoprotein extraction (5). This substance contained N 6.8, P 3.7 per cent. The crude preparation (310 mg.) was taken up in 25 cc. of saline. A large proportion (55 per cent) was insoluble; it contained N 4.1, P 1.9 per cent. From the supernatant the lanthanum salt was prepared and decomposed in the manner described. The *potassium nucleate* (Preparation 2) weighed 29 mg. and was not yet quite pure; it contained N 11.2, P 6.2 per cent. This preparation, containing about 65 per cent of desoxypentose nucleic acid and about 30 per cent of pentose nucleic acid, served for the study of the carbohydrate components included in this paper.

*Desoxypentose Nucleic Acid of Yeast*—The preparation of this material has been described in an earlier communication (9). The sample employed contained N 14.0, P 8.3 per cent.

<sup>1</sup> It might be pointed out that, owing to the greater stability of the pyrimidine nucleotides, the employment of the diphenylamine color reaction (6) for the determination of the purity of a desoxypentose nucleic acid preparation, or for the estimation of desoxypentose nucleic acids in general, requires that the proportion of purines in the nucleic acid serving as the standard and in the unknown be the same. Otherwise, the results cannot be evaluated, unless, as is the case here, the molar proportions of purines are known.

*Composition of Desoxyribose Nucleic Acid of Avian Tubercle Bacilli*

*Preliminary Experiments with 5-Methylcytosine*—In view of the reported presence of this unusual pyrimidine in the nucleic acid of tubercle bacilli (10) it was thought best to perform orienting experiments on the chromatographic behavior of this compound, with a small specimen which, shortly before his death, Professor T. B. Johnson of Yale University had had the goodness to send us. An aqueous solution of the preparation was treated with  $H_2S$ , in order to remove silver which it contained, centrifuged, and concentrated to a small volume under diminished pressure. The chilled concentrate deposited crystals which were collected.

When an aqueous solution of this substance, containing 4.246 mg. (dried in a high vacuum) in 2.5 cc., was subjected, in portions of 0.01 cc., to chromatography on filter paper with *n*-butanol-water as the solvent, it became obvious that it contained two components which, however, were not well separated in the course of 12 hours, the period usually employed for the separation of pyrimidines (3). Extended chromatography for 18 hours brought about the separation of two distinct spots, of which the upper one corresponded to cytosine, both in its absorption maximum and its position on the chromatogram. The lower zone, with an  $R_f$  value approximately 25 per cent greater than that of cytosine, yielded an extract having an absorption maximum at 274  $m\mu$  (in water) and was presumed to represent 5-methylcytosine.

An approximate absorption spectrum of 5-methylcytosine could be constructed by the chromatographic separation of the mixture, the quantitative estimation of the proportion of cytosine present, and the spectroscopy of the second component. A 0.01 cc. portion of the solution (containing 16.98  $\gamma$  of the pyrimidine mixture) was found to yield an average of 7.19  $\gamma$  of cytosine and to correspond, therefore, to 9.79  $\gamma$  of 5-methylcytosine. The extinction values recorded for aqueous extracts of the methylcytosine adsorbates, prepared in the usual way (3), showed an absorption maximum with a center at 274  $m\mu$  and a minimum at about 252  $m\mu$ . At the maximum, the molecular extinction coefficient,  $\epsilon$ , was found to be 10,200.

*Purines and Pyrimidines*—A total of about 7.5 mg. of the nucleic acid (Preparation 1) was available for the complete analysis. For this reason, it was found preferable to perform most of the analyses in a slightly modified sequence that was imposed by the very small quantity available. 7.073 mg. (dried in a high vacuum at 60° for 3 hours) were, in the arrangement previously discussed (4), suspended in 0.5 cc. of absolute methanol and treated with dry HCl gas for 5 hours. Within about 20 minutes the nucleic acid had gone into solution, but it required more than 2 hours for the purine hydrochloride precipitate to make its appearance. The mixture was chilled, the purines were removed by centrifugation,

and the supernatant (containing the pyrimidine fraction) was evaporated and freed of HCl in the manner described before (4).

The purine fraction was dissolved in 0.1 N sulfuric acid (volume of the solution 0.814 cc.) and the solution chromatographed in 0.01 cc. portions (each corresponding to 86.9  $\gamma$  of nucleic acid) with *n*-butanol-diethylene glycol-water (in  $\text{NH}_3$  atmosphere) as the solvent. In contrast to all other nucleic acids examined, only one component, *viz.* guanine, was demonstrable on the developed chromatogram. The amount of *guanine* found per 0.01 cc. of solution was 7.41  $\gamma$  (average of six determinations); *i.e.*, 8.5 per cent of the nucleic acid. No adenine was demonstrated visibly; segments, removed by blind cutting (1, 4), yielded extracts containing traces (about 0.6  $\gamma$ ) of what from its absorption maximum (262  $\text{m}\mu$ ) might have been adenine.

The pyrimidine fraction was hydrolyzed with 0.8 cc. of concentrated formic acid (175°, 2 hours) and processed in the usual manner (4). Its final volume was 0.911 cc. The chromatographic separation was carried out in *n*-butanol-water with 0.03 and 0.04 cc. portions of the hydrolysate (corresponding to 233 and 311  $\gamma$  respectively of the nucleic acid). The development of the chromatograms for pyrimidines demonstrated four distinct spots, two of which were also brought out by the development for purines (3).<sup>2</sup> The pyrimidines were *cytosine*, 6.5 per cent of the nucleic acid (average of six determinations), and *thymine*, 3.0 per cent (eleven determinations). The purines were *guanine*, 1.5 per cent of the nucleic acid, and *adenine*, 3.8 per cent. For the relative position of these compounds on the chromatogram, Fig. 1 of Vischer and Chargaff (3) should be consulted.

As a check on the finding that the treatment with methanolic HCl, which with other nucleic acids (1, 4) effected the liberation of both purine constituents, released only guanine from the nucleic acid of tubercle bacilli, the minute amount of remaining material was subjected to a purine analysis by the customary procedure (4), though with much smaller quantities. 0.404 mg. of the nucleic acid (Preparation 1) was hydrolyzed in 0.1 cc. of N  $\text{H}_2\text{SO}_4$  at 100° for 1 hour in a sealed tube. The hydrolysate was, by means of a capillary pipette, quantitatively transferred to a short paraffin-coated test-tube, 3 drops of  $\text{H}_2\text{SO}_4$  being used for rinsing. The test-tube was kept in an ammonia atmosphere for 15 minutes and then overnight in a non-evacuated desiccator over calcium

<sup>2</sup> The possibility of making use of such differential development procedures should be emphasized. When the chromatograms are developed for purines, *i.e.* via their Hg salts under acidic conditions, eventually present pyrimidines will not be visible. The development procedure for pyrimidines, resting on the production of Hg salts at neutrality, will, however, bring out purines as well (3).

chloride, in order to concentrate the neutralized solution whose final volume was determined, by drawing it into a micrometric burette, as 0.11105 cc. The solution was chromatographed, in 0.011 cc. portions (corresponding to 40.4  $\gamma$  of nucleic acid), with *n*-butanol-diethylene glycol-water (in  $\text{NH}_3$  atmosphere) and separated into *guanine*, 10.3 per cent of the nucleic acid, and *adenine*, 3.8 per cent. The excellent agreement with the figures reported in the preceding paragraph indicates the effectiveness of the estimation procedures, even when performed under difficult conditions and with minute amounts of material.

TABLE I  
*Composition of Desoxypentose Nucleic Acids of Tubercle Bacilli and of Yeast*

Desoxy- pentose nucleic acid	Constituent	Content in nucleic acid	N in nucleic acid	N ac- counted for as per cent of nucleic acid N	Purine N Pyrimidine N	Mole per mole P	P ac- counted for as per cent of nucleic acid P	Average No. of N atoms per con- stituent	Atomic N:P ratio	Molar pro- portions
		<i>per cent</i>	<i>per cent</i>							
Tubercle bacilli	Adenine	3.9	2.0	15.2		0.121	12.1			1.1
	Guanine	10.1	4.7	35.2		0.280	28.0			2.6
	Cytosine	6.8	2.6	19.3		0.256	25.6			2.4
	Thymine	3.2	0.7	5.3		0.106	10.6			1.0
	Total nu- cleic acid			75.0	2.0		76.3	3.9	4.0	
Yeast	Adenine	8.5	4.4	31.5		0.235	23.5			1.8
	Guanine	5.5	2.6	18.2		0.136	13.6			1.0
	Cytosine	3.9	1.5	10.5		0.131	13.1			1.0
	Thymine	8.3	1.8	13.2		0.246	24.6			1.9
	Total nu- cleic acid			73.4	2.1		74.8	3.6	3.7	

Table I includes a summary of the composition of the nucleic acid and of the proportions in which the constituents occur. For the reasons set forth previously (1, 4), the pyrimidine figures were corrected by the addition of a factor of 5 per cent.

*Search for Presence of 5-Methylcytosine*—In no case could a third pyrimidine, in addition to cytosine and thymine, be demonstrated in the hydrolysate of the nucleic acid by the usual development of the guide chromatogram (3). There existed, nevertheless, the possibility of the occurrence of methylcytosine as a minor component. For this reason,

several chromatograms were dissected in such a manner as to cut a narrow cytosine segment (about 2.5 cm. high, 2.7 cm. wide) and directly below, but above the region of adenine adsorption, a second rectangle of the same dimensions, which, in accordance with the preliminary observations on methylcytosine, should have contained this pyrimidine. Each paper segment was extracted with 4 cc. of water and the extracts were read in the spectrophotometer. The results, assembled in Table II, show the absence of an absorbing substance from the region where methylcytosine would have been found. They are particularly con-

TABLE II  
*Search for 5-Methylcytosine in Hydrolysate of Tubercle Bacillus Nucleic Acid*

Wave-length  <i>mμ</i>	Extinction coefficient	
	Cytosine region	Methylcytosine region
300	0.020	-0.011
290	0.046	-0.010
275	0.286	-0.008
267.5	0.322	-0.008
262.5	0.315	-0.008
250	0.262	-0.004
230	0.389	-0.005

vincing, since, in the region directly above, cytosine with the correct absorption maximum, 267.5  $m\mu$ , was found in an amount of 20.2  $\gamma$ .

*Composition of Desoxypentose Nucleic Acid of Yeast*

As the methods previously described (1, 4) were followed, only a few additional remarks are necessary. The purines were liberated by hydrolysis with N sulfuric acid at 100° for 1 hour and separated with *n*-butanol-morpholine-diethylene glycol-water. *Adenine* amounted to 8.5 per cent and *guanine* to 5.5 per cent of the nucleic acid. In another hydrolysis that was carried out for 45 minutes, morpholine was omitted from the solvent mixture and the separation performed in an ammonia atmosphere. The values found here were adenine 8.2 per cent, guanine 5.2 per cent.

In its behavior towards hydrolysis for the determination of pyrimidines the yeast desoxypentose nucleic acid behaved somewhat similarly to the corresponding compound from tubercle bacilli. The purine hydrochlorides appeared 2 hours after the treatment with HCl in methanol had begun. The degradation with methanolic HCl was carried out for a total of 5 hours and the pyrimidine fraction hydrolyzed with formic acid and processed as

usual. Here, too, four adsorption zones were revealed by the development, two belonging to pyrimidines, two to small amounts of purines. The pyrimidines were *cytosine* 3.7 per cent, *thymine* 7.9 per cent of the nucleic acid. The purines consisted of adenine (absorption maximum 262.5  $m\mu$ ), 1 per cent of the nucleic acid, and of a second component, situated above cytosine on the chromatogram, whose solution in  $N$  HCl gave very low absorption readings, with a maximum at 275  $m\mu$ .

The analytical figures employed for the computation of the proportions of the purines and pyrimidines found in this nucleic acid are arranged in Table I.

### *Sugar Components of Microbial Nucleic Acids*

The study of the sugar components of the nucleic acids discussed here utilized procedures that were similar to those followed in this laboratory for the examination of other nucleic acids (1, 4). It was based on the enzymatic cleavage of the nucleic acids to nucleosides, the hydrolysis of the latter with very dilute acid, and the chromatography of the liberated sugars.

The enzymatic digestion of 10 mg. portions of the nucleic acid of tubercle bacilli (Preparation 2) and of the desoxypentose nucleic acid of yeast was carried out with the proportions of enzymes and under the experimental conditions described previously (1). The duration of incubation at 30° was 19 hours, at which time 90 per cent of the organic P of the nucleic acid of tubercle bacillus and 54 per cent of that of the yeast product had been converted to phosphoric acid. The solutions were deproteinized, adjusted to pH 1.3, and heated at 100° for 12 minutes in the manner discussed before (1). The chromatographic study of the hydrolysates with three different solvent mixtures also followed the earlier arrangement.

0.02 cc. portions of the hydrolysate of the tubercle bacillus nucleic acid and 0.03 and 0.05 cc. portions of that of the yeast nucleic acid were subjected to chromatography. An enzymatic digest of thymus nucleic acid (1) served as the reference standard for desoxyribose. Control runs with  $D$ -ribose and with the enzyme mixture alone (with the omission of nucleic acid) also were carried out. Both microbial nucleic acids yielded a sugar component which was identical in its chromatographic behavior in three solvent systems with *2-desoxyribose* from thymus nucleic acid. The tubercle bacillus nucleic acid contained, in addition, ribose as a minor component. The presence of a pentose in this fraction (Preparation 2) was to be expected from the analytical results given at the beginning of this paper, which showed it to be a mixture of desoxypentose and pentose nucleic acids. Lack of material prevented the examination of the

carbohydrate component of the more highly purified Preparation 1 of the tubercle bacillus nucleic acid that had served for the study of the purines and pyrimidines.

The exact composition of the solvent systems has been given in a previous paper (1). The  $R_F$  values (at 30°) of the desoxyribose components of the desoxypentose nucleic acids of yeast, tubercle bacilli, and thymus were as follows: in isobutyric acid 0.55, in butanol-pyridine 0.62, in butanol-ethanol 0.48. The ribose component of the tubercle bacillus product gave, in the same solvents,  $R_F$  values of 0.42, 0.52, 0.32 respectively. In the development of the chromatograms with *m*-phenylenediamine (11), differences in the fluorescent colors of the various sugar bands were observed; whereas that of D-ribose was orange, desoxyribose produced a brilliant, almost white fluorescence. It should be mentioned that the hydrolysate of the yeast desoxypentose nucleic acid showed, in isobutyric acid, a minor, somewhat tailing, slow component with an  $R_F$  of 0.32. Similar bands were also observed with the desoxypentose nucleic acids of thymus and spleen; they possibly represented desoxyribose phosphoric acid.

#### DISCUSSION

The inspection of Table I will show that, with respect to purine and pyrimidine distribution, the microbial nucleic acids differ very much from each other as well as from the desoxypentose nucleic acids of thymus and spleen investigated previously (1). A comparison of the molar proportions reveals certain striking, but perhaps meaningless, regularities.

The composition of nucleic acid preparations from tubercle bacilli has been studied in several instances, but the substances investigated probably were in most cases mixtures of desoxypentose and pentose derivatives. Ruppel (12) reported the presence of thymine, Levene (13) that of thymine and uracil; Long (14) isolated adenine and guanine. Better products, though degraded by alkali, were examined by Johnson and Brown (15, 16). These authors isolated adenine, cytosine, and thymine; guanine was not found in the purified preparation, but could be isolated from the mother liquors accumulated in the course of the purification of the nucleic acid. They concluded that the original nucleic acid was a rather labile substance from which guanine could be split off easily. This inference is not borne out by the present studies which showed guanine to be the major purine constituent. It is probable that the lability of the nucleic acid observed by Brown and Johnson was induced by the treatment with strong alkali. Interesting differences were, however, observed in the present experiments in regard to the ease with which the purines were liberated by methanolic HCl. Whereas in the desoxypen-



tose nucleic acids of animal origin (1) both adenine and guanine were released by this treatment, only the latter compound was set free from the tubercle bacillus nucleic acid. No such differences were found when the liberation of purines was effected with aqueous mineral acid.

The nucleic acid of tubercle bacilli occupies a special place in the literature because of the reported occurrence in it of 5-methylcytosine (10). The evidence was not entirely convincing, resting solely on the crystallographic identification of the picrate, and for this reason, a special search was made for the presence of this pyrimidine in the chromatograms obtained in the course of this study. It was, however, not found. Had it been present in the hydrolysate in a concentration of 0.7 per cent of the nucleic acid (*i.e.* about one-tenth of the amount of cytosine found), it could hardly have escaped detection.

The investigation of the composition of the desoxypentose nucleic acid of yeast presents some interest for a number of reasons. This appears to be the first instance of its study. It also represents, when considered in conjunction with a previous paper (4), the first example of a comparative study by identical methods of the composition of both types of nucleic acid derived from the same cell.

In both microbial desoxypentose nucleic acids discussed here, a certain proportion of the nucleic acid nitrogen (about 25 per cent) has not been accounted for. The possible reasons for these losses have been discussed previously (4), but it should be emphasized that the deficit is somewhat greater in the microbial preparations forming the subject of this paper, and this may well lead to the suspicion that one major constituent or several minor components of these nucleic acids have not yet been identified.

The examination of the carbohydrates contained in the microbial nucleic acids gave interesting results. By their chromatographic behavior in three different solvent systems, the sugars contained in both nucleic acids were shown to be most probably identical with 2-deoxyribose from thymus nucleic acid. The identification must remain tentative as long as the chromatographic properties of other desoxy sugars are not known, but it is unlikely that such parallelism in their positions on the chromatograms could have occurred in three different solvents had the sugars in question not been the same. It has thus been made probable that the desoxypentoses present in the nucleic acids from sources as distinct as thymus, spleen (1), yeast, and tubercle bacilli have their sugar component, or at least one of their sugars, in common, a point very much in doubt until now. It will be understood that, in view of the greater stability of the pyrimidine nucleosides, the presence of other sugars still is conceivable. The carbohydrate of the pentose nucleic

acid of tubercle bacilli has, by similar procedures, been identified with ribose.

The authors are very much indebted to Mrs. Charlotte Green and Miss Ruth Doniger for help in the experiments.

#### SUMMARY

The composition of two desoxypentose nucleic acids derived from avian tubercle bacilli and from yeast was studied. Both compounds were found to contain the purines adenine and guanine and the pyrimidines cytosine and thymine. No 5-methylcytosine could be detected in the nucleic acid of tubercle bacilli. The application of microprocedures permitted the estimation of the major portion of the nitrogenous constituents in minute quantities of these nucleic acids.

The sugar components of both nucleic acids, released by the hydrolysis of the enzymatically produced nucleosides, were in all likelihood identical with the desoxypentose of thymus nucleic acid, *i.e.* 2-desoxyribose. The pentose nucleic acid of tubercle bacilli was shown to contain ribose.

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# ELECTROPHORETIC AND CHEMICAL CHARACTERIZATION OF HUMAN LYMPHOID TISSUE AND CALF THYMUS\*

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(Received for publication, August 30, 1948)

An adequate characterization of protein mixtures extracted from tissues requires, in addition to chemical analysis, the parallel application of the physical techniques developed within recent years and applied so successfully to the analysis of blood plasma. Electrophoretic analysis of cellular extracts, not further manipulated by chemical procedures, have been reported for yeast (1), red cells (2), rabbit muscle (3, 4), rabbit lymphoid tissue (5), mouse lymphosarcoma and calf thymus (6), and dog tissues (7).

In this paper, electrophoretic analyses of extracts of human lymphoid tissue and calf thymus are presented. In addition, the fractions obtained by salt fractionation have been characterized electrophoretically and in part by chemical analysis.

In so far as it was possible, an attempt has been made to preserve the native state of the components of the system by using mild procedures capable of bringing the elements of the cell into a clear solution which could be subjected to electrophoretic analysis. Essentially the procedure consisted of homogenization of the tissue with salt solutions at a neutral or slightly alkaline pH and high speed centrifugation to remove the insoluble material, all operations being carried out in a cold room at 4°.

As a source of "normal" human lymphoid tissue we have used the tonsil, which is available in quantity and offers a means of carrying out a comparative study of human pathological tissue.

Neoplastic lymphoid tissue obtained at autopsy has been of primary interest and several such tissues have been examined, but not in sufficient numbers as yet to allow definite conclusions to be drawn as to significant differences.

Electrophoretic analysis of the tissue proteins permits a direct approach to the problem of the relation between these proteins and those of the blood plasma. The studies of White and Dougherty (5), using this technique, indicated that the lymphocyte might be the source of  $\gamma$ -globulin (antibody globulin), the latter being released from the cell after its dissolu-

\* Aided in part by a grant from the Wisconsin Alumni Research Foundation and in part by a grant from the United States Public Health Service (to Dr. O. O. Meyer, Department of Medicine).

tion under the influence of the adrenal-pituitary hormonal system. Since extracts of human lymphoid tissue and calf thymus appear to contain little or no  $\gamma$ -globulin, as evidenced by the electrophoretic patterns and fractionation studies, the rôle of the lymphocyte as the principal source of plasma  $\gamma$ -globulins would appear to be doubtful.

### Methods

Electrophoresis was carried out in the Tiselius apparatus as modified by Longworth (8). All runs were made in veronal buffer (after dialysis to equilibrium), 0.1 ionic strength, pH 8.60, at 1.0°. Mobilities were calculated from the distance from the starting position to the maximum ordinate of each peak in the electrophoretic diagram. The areas were determined from tracings of the enlarged pattern, and the same specific refractive increment was assumed for each component. For the purpose of measuring the relative concentrations of the components, the electrophoretic diagram was divided by means of dropping a perpendicular line from a readily observable separation to the base-line, as suggested by Tiselius and Kabat (9). Calculations of mobility and relative concentration were made only from patterns of the descending limb. A tungsten lamp light source used in conjunction with filters proved very useful in obtaining patterns of strongly opalescent solutions. The voltage gradients and times of electrophoresis are indicated in Figs. 1 to 3.

Total nitrogen was determined by the micro-Kjeldahl method. Total phosphorus was determined by the method of King (10). The Mejbaum procedure as modified by Schneider (11) was used for the determination of ribonucleic acid.<sup>1</sup> The presence of desoxyribonucleic acid was tested in hot trichloroacetic acid extracts by the Dische diphenylamine reaction as described by Seibert *et al.* (12). Total cholesterol was determined colorimetrically by the Liebermann-Burchard reaction after extraction of the dry protein fractions with hot alcohol-ether (3:1). Lipide phosphorus was determined on an aliquot of the hot alcohol-ether extract.

### EXPERIMENTAL

#### *Human Lymphoid Tissue (Tonsil)*

Tonsil tissue obtained as soon as possible from the operating room was cut into thin slices with a razor blade and washed by repeated suspension in 0.14 M NaCl solution and centrifugation in order to remove as much as

<sup>1</sup> Actually ribose is determined and assumed to be derived from ribonucleic acid. Ribonucleic acid (Schwarz), whose purity was determined from its phosphorus content, was used as a standard. Likewise, purified desoxyribonucleic acid of known phosphorus content was used as a standard.

possible of contaminating extracellular fluids (blood, lymph, etc.). The washed tissue was then stored in a deep freeze unit at  $-30^{\circ}$  for pooling if large amounts were required for fractionation studies. If the individual tonsil was to be studied, it was immediately homogenized in a Waring blender<sup>2</sup> and centrifuged. An individual tonsil pair from a young adult weighs approximately 10 gm. Homogenization (3 to 5 minutes) with twice its weight of salt solution yielded more than sufficient clear supernatant fluid for electrophoretic analysis in the conventional Tiselius cell. These extracts contained approximately 2 per cent protein. The centrifugation was carried out in a Sorvall angle centrifuge, model SS-1, for 2 hours at approximately 15,000*g*.

Two types of extracts may be differentiated: (a) one in which the extracting medium is a dilute salt solution, approximately 0.14 M (this extract contains no significant amount of desoxyribonucleic acid); (b) one in which

TABLE I

*Percentage Distribution of Nitrogen and Phosphorus in Extracts and Residues of Human Tonsil and Calf Thymus (Extracted with 0.14 M NaCl Solution)*

		Insoluble	Soluble	
			Non-dialyzable	Dialyzable
Tonsil	Nitrogen	50	39	11
	Phosphorus	61	14	25
Calf thymus	Nitrogen	51	37	12
	Phosphorus	62	8.2	30

the extracting medium is high in salt concentration, *viz.* 10 per cent NaCl (this extract contains large quantities of desoxyribonucleic acid).

*Extraction with Dilute Salt Solutions*—The per cent distribution of nitrogen and phosphorus among the insoluble, soluble non-dialyzable, and soluble dialyzable fractions of the tissue is shown in Table I. These figures were obtained by taking aliquots from the whole tissue homogenate, from the supernatant after high speed centrifugation, and from the supernatant after dialysis. The absence of desoxyribonucleic acid in any significant amount from this extract indicates that its contents are derived, for the most part, from the cytoplasm of the tissue cells. Mirsky and Pollister have described similar findings for calf thymus (13).

An electrophoretic pattern typical of these tonsil extracts is shown in Fig. 1, *a* and *b*. The mobilities and the relative percentage composition of components obtained from the analysis of a large number of individual tonsils are shown in Table II. The numbers over each electrophoretic

<sup>2</sup> Microscopic examination showed only a few cells to be left intact.

pattern designate components or groups of components whose mobilities are listed under the corresponding numbers in Table II. None of the quali-

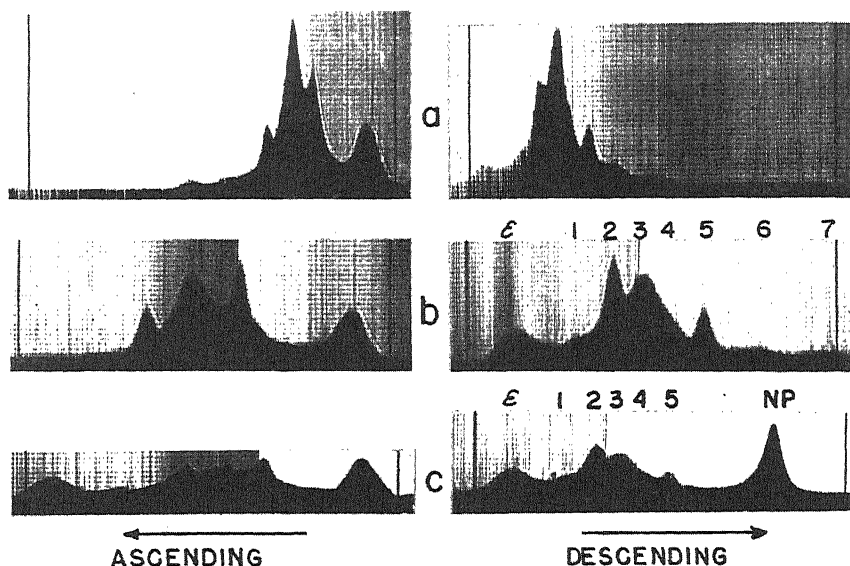


FIG. 1. Electrophoretic patterns of the whole extract of human tonsil. *a*, extracted with 0.07 M  $\text{KHCO}_3$ -0.07 M KCl solution, pH 8.0; electrophoresis for 80 minutes at 5.8 volts per cm. *b*, same as *a* but electrophoresis for 150 minutes at 5.8 volts per cm. *c*, extracted with 10 per cent NaCl solution; electrophoresis for 126 minutes at 5.8 volts per cm.

TABLE II

*Mobility and Relative Percentage Composition of Electrophoretic Components in Extracts\* of Human Lymphoid Tissue (Tonsil)†*

	Component 1	Component 2	Component 3	Component 4	Component 5	Component 6	Component 7
Mobility‡	2.09 ±0.26	3.54 ±0.26	4.79 ±0.32	5.64 ±0.34	6.89 ±0.34	9.1 ±0.3	11.9 ±0.6
Relative % composition	5.88 ±2.1	27.2 ±5.7	48.0 ± 9.5		13.2 ±1.8	6.0 ±1.8	3.6 ±1.0

\* Extracting medium, physiological salt solution.

† The standard deviation of fifteen determinations is given.

‡  $10^5$  sq. cm. per volt per second in veronal buffer, pH 8.6, 0.1 ionic strength. All components migrate toward the anode.

tative features of the electrophoretic pattern was altered by variation of the type of salt and the pH used for extraction, provided the salt con-

centration was close to 0.14 M. Thus the same pattern was obtained with the following as extracting agents: 0.14 M NaCl, unbuffered, final pH of extract, 6.8; 0.14 M NaCl-0.02 M potassium phosphate, pH 7.0; 0.07 M KCl-0.07 M  $\text{KHCO}_3$ , pH 8.0; Na veronal, 0.12 M, pH 8.6. The KCl- $\text{KHCO}_3$  extracting medium is probably most advantageous, since its higher alkalinity not only insures greater solubility of the tissue proteins, but also permits analysis of different phosphate fractions. In addition, the high pH inhibits the catheptic enzymes as pointed out by Greenstein (14). However, because of the loss of  $\text{CO}_2$  the pH of this system usually rises from an initial value of approximately 8.0 to 8.2 to 8.3.

*Extraction of Tonsil with 10 Per Cent NaCl Solution*—In contrast to the behavior of the tissue when dilute salt solution is used for extraction, 10 per cent NaCl causes large amounts of desoxyribonucleic acid to be brought into solution (15). Correspondingly, a large amount of a fast component (component NP in Fig. 1, c) appears in the electrophoretic pattern, superimposed on the other slower components which are apparently the same cytoplasmic proteins observed previously. The pattern is shown in Fig. 1, c. The fast component (desoxyribonucleoprotein) has a mobility of  $-11.8$  sq. cm. per volt per second. It probably corresponds to the calf thymus nucleohistone characterized by Hall (16). It seems important to point out, however, that this fast component does not precipitate on being brought back to dilute salt concentration (dialysis against veronal buffer, 0.1 ionic strength, pH 8.6). It will be recalled that direct extraction of the tissue with 0.1 ionic strength veronal does not cause this component to appear in the electrophoretic pattern. It may be inferred, therefore, that treatment with 10 per cent NaCl solution has altered, irreversibly, the native state of the desoxyribonucleoprotein from the point of view of its solubility. One should not, however, exclude the possibility that the alteration was brought about by the action of a depolymerase acting during the period in which the substrate was in solution.

### *Calf Thymus*

Extracts of fresh calf thymus were prepared in the same manner as described for tonsil, with 0.07 M KCl-0.07 M  $\text{KHCO}_3$  solution. The percentage distribution of nitrogen and phosphorus in the insoluble matter, the soluble non-dialyzable fraction, and the soluble dialyzable fraction are shown in Table I. A typical electrophoretic pattern and analysis are shown in Fig. 2, a and Table III respectively.

### *Fractionation of Tonsil and Calf Thymus Extracts*

From extracts of pooled tonsil and fresh calf thymus, ammonium sulfate fractions were obtained at 30 per cent saturation ( $4^\circ$ ), 30 to 55 per cent

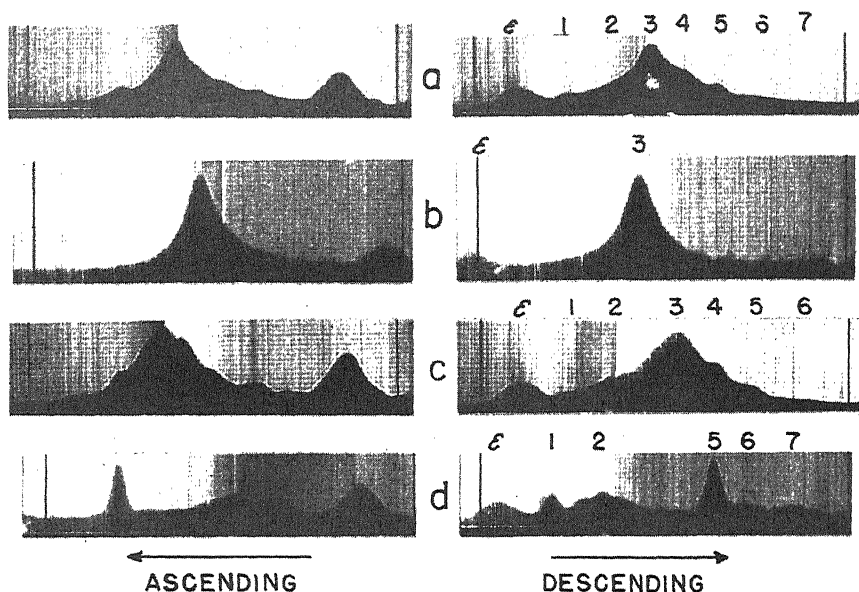


FIG. 2. Electrophoretic patterns of calf thymus fractions. The method of preparation is as described in the text. *a*, whole extract, electrophoresis for 150 minutes at 5.8 volts per cm.; *b*, Fraction 30P, electrophoresis for 180 minutes at 5.9 volts per cm.; *c*, Fraction 55P, electrophoresis for 180 minutes at 5.8 volts per cm.; *d*, Fraction 100P, electrophoresis for 170 minutes at 5.8 volts per cm.

TABLE III  
*Electrophoretic Analysis of Calf Thymus Extract and  $(\text{NH}_4)_2\text{SO}_4$  Fractions*

Fraction No.		Component 1	Component 2	Component 3	Component 4	Component 5	Component 6	Component 7
Whole extract	Mobility*	2.1	3.6	4.9	5.9	7.0	8.1	9.5
	% composition	6.5	18	38	20	8.8	5.0	2.6
30P	Mobility			4.7				
	% composition			100 (ca.)				
55P	Mobility	1.6, 2.3	3.0, 3.7	5.1	6.4	7.4	9.0	
	% composition	3.7, 6.1	9.9	57	14	8.2	2.0	
100P	Mobility	1.5	2.7, 3.2, 3.8			7.1	8.2	9.6
	% composition	14	9, 14, 12			30.5	7.2	7.4

\*  $10^5$  cm. sq. per volt per second, in veronal buffer, pH 8.6, 0.1 ionic strength. All components migrate toward the anode.

saturation, and 55 to 100 per cent saturation. The salt was added by immersing a cellophane bag containing the extract into the appropriate



concentration of salt and allowing diffusion to equilibrium to take place. The pH was maintained close to neutrality by the addition of  $\text{NH}_4\text{OH}$ . The precipitate formed at 30 per cent saturation (Fraction 30P) was removed by centrifugation and washed free of contaminating supernatant, with the outside equilibrium dialysis fluid as the wash medium. The supernatant was then reimmersed and brought to equilibrium with 55 per cent saturated ammonium sulfate, whereupon the precipitate which formed was separated (Fraction 55P) and washed with the outside fluid. The same process was repeated to obtain the fraction precipitating between 55

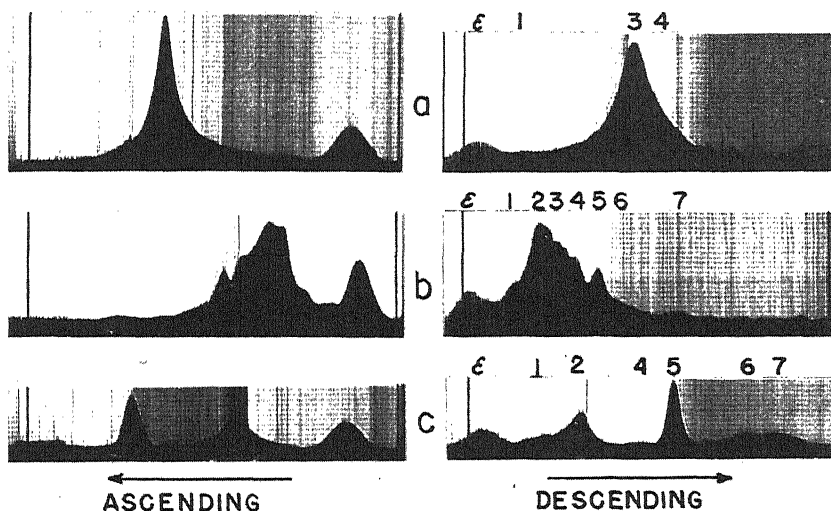


Fig. 3. Electrophoretic patterns of human tonsil fractions. The method of preparation is as described in the text. *a*, Fraction 30P, electrophoresis for 182 minutes at 5.7 volts per cm.; *b*, Fraction 55P, electrophoresis for 104 minutes at 5.7 volts per cm.; *c*, Fraction 100P, electrophoresis for 150 minutes at 6.1 volts per cm.

and 100 per cent saturation of salt (Fraction 100P). Only the fraction obtained at 30 per cent saturation failed to dissolve completely in the  $\text{KCl-KHCO}_3$  solution. Thus a fourth insoluble fraction was obtained (Fraction 30P-Ins). Of the total protein in the extracts, Fraction 30P represents approximately 50 per cent, Fraction 55P, 15 per cent, and Fraction 100P, 10 per cent.

The electrophoretic patterns and analyses for each soluble fraction are shown in Fig. 2, *b*, *c*, and *d* and Table III for calf thymus, and Fig. 3, *a*, *b*, and *c* and Table IV for human tonsil.

#### *Chemical Properties of Soluble "Cytoplasmic" Proteins*

The soluble "cytoplasmic" proteins are associated with small amounts of phosphorus, most of which is in the form of phospholipide and ribonucleic

acid. Significant quantities of cholesterol are also present. An analysis of the protein fractions previously mentioned is given in Table V. These fractions contain no significant quantity of desoxyribonucleic acid.<sup>3</sup> It will be noted that the thymus Fraction 30P-Ins, the portion of the precipi-

TABLE IV  
*Electrophoretic Data on (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> Fractions of Human Tonsil Extracts*

Fraction No.		Component 1	Component 2	Component 3	Component 4	Component 5	Component 6	Component 7
30P	Mobility*	1.8		4.7	5.0			
	% composition	3		83	9			
55P	Mobility	2.2	3.5	4.5, 4.8	5.5	6.8	7.9	11.3
	% composition	9.1	37	19	13	12.5	7.0	2.4
100P	Mobility	1.4, 2.1	2.7, 3.4		5.5	6.8	9.3	10
	% composition	2.7, 5.0	6.4, 23		2.3	32		28

\* 10<sup>5</sup> sq. cm. per volt per second, in veronal buffer, pII 8.6, 0.1 ionic strength. All components migrate toward the anode.

TABLE V  
*Chemical Analysis of Fractions\* from Human Tonsil and Calf Thymus Extracts*  
The results are expressed in per cent.

	Fraction No.	Nitrogen	Phosphorus	Phospholipide†	Total cholesterol	Ribonucleic acid
Tonsil	30P	13.2	0.44			1.6
	55P	13.7	0.26			1.1
	100P	14.1	0.40			2.1
Calf thymus	30P	13.9	0.55	3.6	1.4	2.3
	55P	14.0	0.50	3.8	2.2	1.0
	100P	14.9	0.21	1.0	0.3	0.6
	30P-Ins	10.9	1.10	11.0	1.5	3.3

\* Dried by lyophilization and redried at 105° for 2 hours previous to weighing.

† Calculated from alcohol-ether-soluble phosphorus multiplied by 25. Phospholipide and cholesterol were not determined quantitatively in tonsil fractions, but qualitative tests show their presence.

tate obtained at 30 per cent saturation of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> which does not redissolve, contains a large amount (11 per cent) of phospholipide.

#### RESULTS AND DISCUSSION

The complexity of the mixture in the whole extract is apparent from an

<sup>3</sup> Mirsky and Pollister (13) have pointed out that autolysis causes the appearance of desoxyribonucleic acid in physiological saline extracts of calf thymus. We have found this to be true for tonsil tissue. Indeed, electrophoretic analysis of a partially autolyzed tonsil (after standing 10 days at 0°), extracted with 0.14 M NaCl, shows a large fast component and gives a very strong reaction with diphenylamine.

examination of the electrophoretic pattern. Nevertheless, definite groups of proteins can be realized and defined by their mobilities. The reproducibility of the patterns of tonsil extracts from a large number of "uncontrolled" human subjects would indicate that the observed electrochemical protein distribution is inherently characteristic of the cells (predominantly lymphocytes) which make up the tissue. It would seem worth while, therefore, to use such patterns as a base-line for the study of human pathological lymphoid tissue, and such a study is in progress.

In the region of the electrophoretic pattern where one would expect to find a component corresponding to plasma  $\gamma$ -globulin (antibody globulin), one observes only a very small non-definitive refractive index gradient (Fig. 1, *a* and *b*). Furthermore, the pattern for the fraction precipitating at 30 per cent saturation of ammonium sulfate shows no evidence of a plasma  $\gamma$ -globulin component, Fig. 2, *b* and Fig. 3, *a*. Inasmuch as plasma  $\gamma$ -globulin is almost completely precipitated<sup>4</sup> by 35 per cent saturated ammonium sulfate (17, 18), it can be inferred that, if this component is present at all, the amount is too small to be detected by this technique. It will also be noted that the components of slow mobility tend to become concentrated in Fraction 100P (Fig. 2, *d*, Fig. 3, *c* and Tables III and IV). This behavior would not be expected of plasma  $\gamma$ -globulin.

Component 5 (Tables III and IV) has *both* the mobility and solubility properties of plasma albumin. It is possible that this "albumin" component is present as a contaminant from plasma and lymph, appearing despite repeated washing of the sliced tissue before the cells are broken. On the other hand, the possibility is not excluded that this component is a true intracellular albumin, or even identical with plasma albumin, and in equilibrium with the albumin of the lymph fluid which bathes the cells.

A small quantity of contaminating hemoglobin becomes concentrated in Fraction 100P (Fig. 2, *d*, Fig. 3, *c*) and appears with a component of mobility of  $-3.4$  sq. cm. per volt per second.

Chemical analysis shows that small amounts of ribonucleic acid are present. By means of an electrophoretic separation experiment, the fast component of mobility of  $-12$  sq. cm. per volt per second can be shown to be ribonucleic acid, migrating free of protein. One cannot say whether ribonucleic acid actually exists as a free entity in the cell or appears as a result of decomposition of the nucleoprotein complexes (mitochondria, microsomes) in the preparative process.

The bulk of the intracellular soluble protein falls into a narrow mobility range and the components do not readily resolve. Even though Fraction 30P of both calf thymus and tonsil shows only one principal peak (Fig. 2, *b*, Fig. 3, *a*), its inhomogeneity is evident from the spreading effect with

<sup>4</sup> It has been observed in this laboratory that the  $\gamma$ -globulin of human serum diluted 1:40 is 50 per cent precipitated by 30 per cent saturated ammonium sulfate.

increased time of electrophoresis. Fractions 55P and 100P are obviously made up of many electrochemical entities.

The system we are dealing with from the electrophoretic standpoint seems to be as complex as the blood plasma, if not more so. The fractionation scheme was devised, not for the purpose of isolating any given component, but to gain information concerning the over-all properties of the intracellular proteins in the hope that a rational basis might be provided for a systematic separation of these proteins. The comparison of *both* the solubility and electrophoretic properties of the tissue protein and serum proteins has provided some basis for studying the relationship between tissue and plasma proteins. In addition to the relationship already discussed ( $\gamma$ -globulin, albumin), it can be seen from an examination of Tables III and IV that Fraction 55P of both tonsil and calf thymus contains proteins with roughly the same solubility and mobility<sup>5</sup> as the serum  $\alpha$ - and  $\beta$ -globulins and, in addition, contains a considerable amount of cholesterol and phospholipide (Table V). These are also properties of the plasma  $\alpha$ - and  $\beta$ -globulins (19). If one neglects the possibility of a fortuitous correspondence in properties, it would seem reasonable to suggest that plasma  $\alpha$ - and  $\beta$ -globulins (but not the  $\gamma$ -globulins) may be derived in part from lymphoid tissue.

The authors wish to express their thanks to Professor O. O. Meyer, Department of Medicine, for his cooperation and interest in this investigation.

#### SUMMARY

1. The cytoplasmic proteins extracted from human lymphoid and calf thymus tissue have been examined electrophoretically. Conditions for realizing reproducible patterns have been established.

2. Extracts of human lymphoid tissue and calf thymus have been fractionated by means of  $(\text{NH}_4)_2\text{SO}_4$  and the fractions characterized electrophoretically and in part by chemical analysis.

3. A comparison has been made of the mobilities, solubilities, and chemical properties of the proteins of plasma and lymphoid tissue extracts. The possible interrelationship of these proteins has been discussed.

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<sup>5</sup> The component with a mobility of  $-4.7 \times 10^{-5}$  sq. cm. per volt per second is responsible for the opalescence of the tissue extracts, while the  $\beta$ -globulin of serum, mobility of  $-2.9 \times 10^{-5}$  sq. cm. per volt per second, is the opalescent component in serum.

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# GROWTH-PROMOTING ACTIVITIES OF GUANINE, GUANOSINE, GUANYLIC ACID, AND XANTHINE FOR A PURINE-DEFICIENT STRAIN OF *NEUROSPORA*\*

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(Received for publication, June 14, 1948)

Biological assays of ribonucleic acid hydrolysates for adenine with the adenine-deficient *Neurospora* mutant No. 28610 have given higher values (1, 2) than those found by other methods. It has also been shown that of the known purine and pyrimidine compounds only adenine and hypoxanthine, when added as the sole supplement to an otherwise adequate medium, promoted growth of the mold (1). Two possible explanations for these apparent discrepancies have suggested themselves; namely, (a) that adenine or some other compound with growth-promoting activity was present in ribonucleic acid in a form not determined by the usual methods of analysis or (b) that one or more of the other purine or pyrimidine components could be utilized for growth in the presence of adenine. Experiments with an equimolar mixture of the four ribonucleotide components of ribonucleic acid showed a growth-promoting activity about twice that expected from the adenine present. Further experiments with pairs of the four nucleotides before and after hydrolysis showed that the increased growth activity was restricted to the mixture of guanylic acid and adenylic acid and to their hydrolytic products. A systematic study of the effect of guanine, guanosine, guanylic acid, and xanthine on growth of the mold in the presence of adenine, of xanthine and guanine when added in addition to hypoxanthine, and of guanosine in the presence of adenosine was undertaken. The results of these experiments are presented in this paper.

## EXPERIMENTAL

*Methods*—The supplementary effect of guanine and its derivatives on the growth of *Neurospora* mutant 28610 was determined by growing the mold in a basal medium containing constant amounts of adenine, adenosine, or hypoxanthine, each of which directly supports growth of this mutant, and adding varying amounts of the substances being tested. The amount of growth was measured by the average dry weight of mycelium produced after 72 hours at 25° in triplicate experiments. The composition of the

\* Aided by a grant from the Rockefeller Foundation.

basal medium and the method of maintaining the cultures and harvesting the mycelium have been described previously (1, 3).

*Growth Curves of Mutant Strain 28610 on Adenine, Adenosine, and Hypoxanthine*—The growth curves previously described for this mutant were based on an incubation period of 84 hours (1). In the present experiments a period of 72 hours was used. This gave reproducible results and avoided the larger amounts of conidia which are produced by this mutant

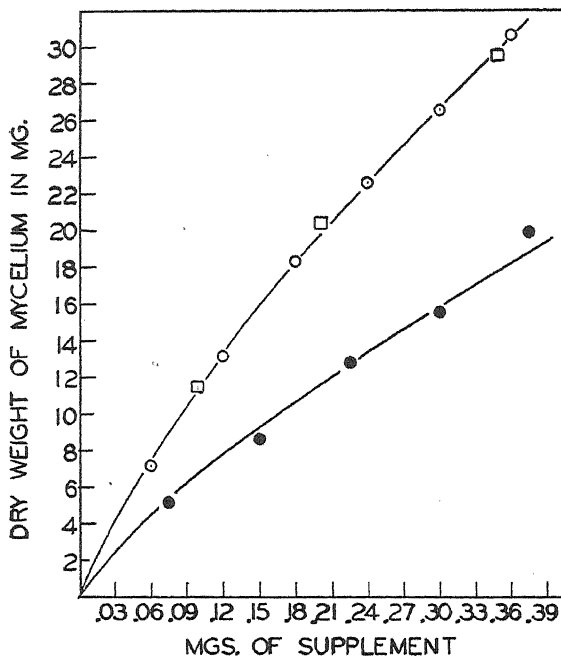


Fig. 1. Growth curves for *Neurospora* mutant 28610 on adenine (O), hypoxanthine (□), and adenosine (●), in 25 ml. of basal medium for an incubation period of 72 hours.

after the longer period of growth. Since growth curves under these conditions have not been published before, they are presented for adenine, adenosine, and hypoxanthine in Fig. 1. Portions of a standard solution of each compound were added to 25 ml. of basal medium and the growth at each concentration was determined. As shown in Fig. 1, the growth responses to adenine and hypoxanthine were essentially the same. Adenosine was utilized equally efficiently when compared on a molar basis.

*Supplementary Growth Effect of Guanine, Guanosine, and Guanylic Acid in Presence of Adenine*—The supplementary effect of guanine on the growth



of the mold in the presence of adenine was determined by adding varying amounts of guanine to a series of flasks containing 25 ml. of basal medium and 0.1 mg. of adenine. The molar ratios of guanine to adenine used were 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 2.5, and 3.0. The weights of mycelium found for the different ratios, their corresponding values expressed as mg. of adenine as given by the adenine growth curve, and the ratios of the adenine values found to the amount of adenine used are summarized in Table I.

Within the limits of accuracy of the method those flasks in which the ratio of guanine to adenine ranged from 0.25 to 1.0 gave the same weight of mycelium in each case as would have been obtained by the addition of adenine itself in an equivalent amount. Thus as long as an equimolar ratio of guanine to adenine was not exceeded, the growth produced by mix-

TABLE I

*Growth-Promoting Activity of Guanine in Presence of 0.1 Mg. of Adenine per 25 Ml. of Basal Medium*

Guanine added	Molar ratio, guanine to adenine	Dry weight of mycelium	Growth activity expressed as adenine	Ratio of growth activity as adenine to adenine present
<i>mg.</i>		<i>mg.</i>	<i>mg.</i>	
0	0	11.2	0.099	0.99
0.028	0.25	13.5	0.125	1.25
0.056	0.50	15.2	0.143	1.43
0.084	0.75	18.5	0.178	1.78
0.112	1.00	19.6	0.192	1.92
0.168	1.50	20.3	0.200	2.00
0.224	2.00	20.3	0.200	2.00
0.280	2.50	24.3	0.257	2.57
0.336	3.00	24.4	0.257	2.57

tures was proportional to the total equivalents of purine, regardless of the actual proportion of the two compounds present. In the flasks containing ratios of guanine to adenine of 1.5 and 2.0, the growth was the same as that found for an equimolar ratio. Thus over the range from 1.0 to 2.0 equivalents of guanine, a plateau of growth was obtained at a level corresponding to approximately twice the amount of adenine actually present. 2.5 and 3.0 equivalents of guanine gave a further increase in the weight of mycelium produced, resulting in a second plateau at a level equivalent to about 2.6 times the adenine used.

The results described above for mixtures of adenine and guanine differ quantitatively from those described in a preliminary report (4) in that the plateau of growth was reached at a higher level and at a higher guanine to adenine ratio. The error in the previous results was later found to be due to the use of a basal medium containing less than an optimum amount of biotin.

The effect of guanosine and guanylic acid upon growth in the presence of adenine was determined as in the experiments just mentioned. The amount of adenine in each flask and the molar ratios of supplement to adenine were the same as those previously used for guanine. The results, which are shown in Table II, were similar to those found with mixtures of adenine and guanine. The increased growth, expressed as mg. of adenine, was directly proportional to the equivalents of supplement used over the range from 0.25 to 1.0 mole of guanosine or guanylic acid per mole of adenine. When the ratio of either compound to adenine was increased from 1 to 3 moles, no further stimulation of mycelial growth took place. Thus with guanosine and guanylic acid a plateau of growth similar to that

TABLE II

*Growth-Promoting Activity of Guanosine and Guanylic Acid in Presence of 0.1 Mg. of Adenine per 25 Ml. of Basal Medium*

Molar ratio, supplement to adenine	Guanosine			Guanylic acid		
	Dry weight of mycelium	Growth activity expressed as adenine	Ratio of growth activity as adenine to adenine present	Dry weight of mycelium	Growth activity expressed as adenine	Ratio of growth activity as adenine to adenine present
	mg.	mg.		mg.	mg.	
0	11.3	0.100	1.00	11.2	0.099	0.99
0.25	13.7	0.128	1.28	13.6	0.125	1.25
0.50	15.4	0.151	1.51	15.9	0.150	1.50
0.75	17.4	0.175	1.75	17.5	0.168	1.68
1.0	18.8	0.195	1.95	19.6	0.192	1.92
1.5	18.1	0.185	1.85	20.4	0.202	2.02
2.0	18.6	0.192	1.92	19.7	0.193	1.93
2.5	18.5	0.191	1.91	19.5	0.191	1.91
3.0	18.9	0.196	1.96	20.1	0.199	1.99

observed with adenine and guanine was also found. The total stimulation of growth in this case, however, did not exceed twice the value found for growth on adenine alone.

*Supplementary Growth Effect of Guanosine in Presence of Adenosine*

In the experiments with adenosine and guanosine, 0.15 mg. of adenosine was added to each 25 ml. of basal medium to provide about the same amount of mold growth as that obtained in the previous experiment with 0.1 mg. of adenine. Amounts of guanosine were then added to each flask to give molar ratios of 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 2.5, and 3.0 to the adenosine present. Increased growth above that expected from the 0.15 mg. of adenosine was obtained with all concentrations of guanosine used. As in the previous experiments with adenine, molar ratios of guanosine to adenosine of 1.0 or less gave increased growth which, in terms of adenosine,

was directly proportional to the amount of guanosine added. As the amount of guanosine was increased above the equimolar ratio, a gradual increase in growth resulted until a plateau was reached over the range from 2 to 3 equivalents of guanosine. The amount of growth found with the latter ratios corresponded to about 2.5 times the value for the adenosine present.

*Supplementary Growth Effect of Xanthine on Adenine*—Amounts of xanthine were added to 0.1 mg. of adenine in 25 ml. of basal medium to give molar ratios over the range from 0.25 to 3.0 moles of xanthine per mole of adenine. The results, which are given in Table III, show that within experimental error the same amount of growth was obtained in all cases,

TABLE III

*Growth-Promoting Activity of Xanthine in Presence of 0.1 Mg. of Adenine or 0.1 Mg. of Hypoxanthine*

Molar ratio, xanthine to adenine (or hypoxanthine)	Adenine + xanthine			Hypoxanthine + xanthine		
	Dry weight of mycelium	Growth activity as adenine	Ratio of growth activity as adenine to adenine present	Dry weight of mycelium	Growth activity as hypoxanthine	Ratio of growth activity as hypoxanthine to hypoxan- thine present
	mg.	mg.		mg.	mg.	
0	11.3	0.100	1.00	11.6	0.103	1.03
0.25	13.7	0.127	1.27	13.3	0.123	1.23
0.50	14.5	0.135	1.35	16.3	0.155	1.55
1.00	13.3	0.122	1.22	16.5	0.157	1.57
1.50	13.8	0.128	1.28	16.1	0.152	1.52
2.00	13.2	0.121	1.21	16.3	0.155	1.55
2.50	13.6	0.126	1.26	15.9	0.150	1.50
3.00	13.5	0.125	1.25	14.3	0.132	1.32

an amount equivalent to about 1.25 times that expected from the adenine alone.

*Supplementary Growth Effect of Xanthine in Presence of Hypoxanthine*—To determine the supplementary effect of xanthine when the mold was provided with hypoxanthine, experiments similar to those described previously were performed. Amounts of xanthine ranging from 0.25 to 3.0 moles were added to 25 ml. of basal medium containing 0.1 mg. of hypoxanthine, and the growth-promoting ability of these solutions determined. The addition of xanthine to hypoxanthine, as in the case of the adenine experiments, resulted in further stimulation of growth. A surprising difference was found, however, in that xanthine was better utilized for growth in the presence of hypoxanthine than in the experiments with adenine. Over the range from 0.25 to 0.50 mole of xanthine per mole of hypoxanthine, as shown in Table III, the increase in the weight of mycelium was

proportional to the amount of xanthine added. As the ratio of xanthine to hypoxanthine was increased beyond 0.5, no further increase in growth resulted and the maximum value corresponding to about 1.5 times the response to hypoxanthine alone remained relatively constant up to 3 moles of xanthine per mole of hypoxanthine. As mentioned above, the maximum growth reached in the experiments in which adenine was supplemented with xanthine was 1.25 times that of adenine alone. It is evident,

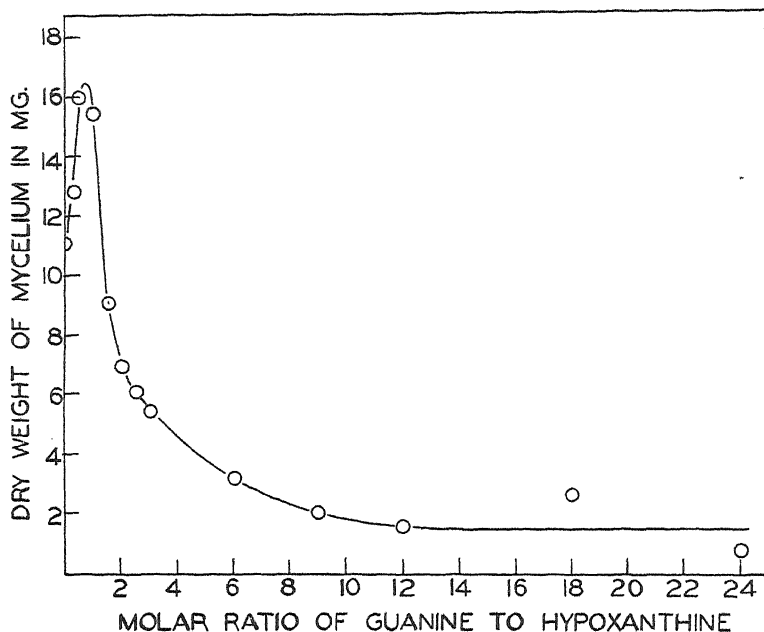


FIG. 2. Supplementary growth and inhibitory effects of guanine upon *Neurospora* mutant 28610 in the presence of 0.1 mg. of hypoxanthine per 25 ml. of basal medium.

therefore, that xanthine is more available for growth when added to hypoxanthine than to adenine.

*Supplementary Growth and Inhibitory Effects of Guanine on Hypoxanthine*—Experiments similar to those already mentioned were performed with mixtures of guanine and hypoxanthine. The amount of hypoxanthine used in each flask was 0.1 mg. and the amount of guanine ranged from 0.25 to 24 moles of guanine per mole of hypoxanthine. The results are shown graphically in Fig. 2, in which the weights of mycelium obtained were plotted against the molar ratios of guanine to hypoxanthine used. Increasing amounts of growth were obtained with low concentrations of guanine,

reaching a maximum of about 1.6 times the value for hypoxanthine alone, when the ratio of guanine to hypoxanthine was 0.75. When the ratio was increased beyond 1, definite inhibition resulted. Complete inhibition was not found even with a ratio as high as 24:1. Once the inhibiting level was reached, as shown in Fig. 2, the amount of growth was roughly inversely proportional to the guanine-hypoxanthine ratio.

#### DISCUSSION

Since the *Neurospora* mutant 28610 fails to grow on a basal medium supplemented with guanine but grows at a normal rate in the presence of moderate amounts of adenine, it was originally assumed that the failure of growth was due to an adenine deficiency, and that guanine was probably synthesized by an independent pathway. The present finding that better growth is obtained when guanine is supplied in addition to adenine provides evidence that this mold is also deficient in the ability to synthesize guanine from simple constituents. When growth is obtained on adenine alone, it now seems probable that the latter is in part converted to guanine and that guanine biosynthesis in this strain of *Neurospora* is analogous to that reported by Brown, Roll, and Plentl for the white rat (5). In the experiments reported by the latter workers guanine containing labeled nitrogen as well as labeled adenine was recovered from the nucleic acid fractions isolated after the feeding of labeled adenine. In agreement with the earlier findings of Plentl and Schoenheimer (6), labeled guanine, however, was not converted to adenine and in fact was not utilized for nucleic acid synthesis. It appears that in both this strain of *Neurospora* and in the rat adenine may be converted irreversibly to guanine, but that only in the former case is guanine utilized for nucleic acid synthesis. It should be emphasized, however, that the utilization of guanine for growth in the present experiments does not constitute proof of its incorporation into the structure of mold nucleic acid. In connection with the latter question, it is of interest that the amount of growth in the presence of a constant concentration of adenine reached a relatively constant value when the ratio of guanine to adenine, either in the free or combined form, was 1. However, better growth resulted in the guanine-adenine and in the guanosine-adenosine mixtures when twice as much guanine as adenine was used.

The increased growth produced by the addition of xanthine to adenine and to hypoxanthine is readily explained by reconversion of xanthine to guanine or to hypoxanthine by the mold. The equilibrium for the guanine-xanthine reaction does not appear to have been studied quantitatively, but such an equilibrium is known in the case of the hypoxanthine-xanthine-uric acid reaction with xanthine oxidase from milk (7). The poor utilization of xanthine for growth compared to guanine indicates that it may be more

subject to degradative reactions. The difference in growth stimulation found when xanthine was added to adenine, as compared to hypoxanthine, suggests that xanthine may inhibit adenine utilization or conversion to guanine or hypoxanthine.

The inhibition of hypoxanthine utilization by guanine after stimulation of growth at lower guanine levels seems to involve the amination of hypoxanthine to adenine, for this is the only additional reaction necessary when the mold grows on hypoxanthine in the presence of guanine. The evidence that this inhibition is a function of the guanine-hypoxanthine ratio suggests the possibility of competition for an enzyme system by these structurally related compounds. Similar blocking of essential reactions has been shown for other components of nucleic acids in the case of the inhibition of growth of the pyrimidine-deficient *Neurospora* mutant 1298 by adenosine or adenosine-3-phosphate (8). The combined results strengthen the suggestion that a mobile equilibrium between the components of the nucleic acids may function in the control of growth in other organisms as well.

It should be noted that *Neurospora* mutant 28610 can still be used in the bioassay of nucleic acids for adenine. As xanthine and hypoxanthine probably exist in nucleic acid hydrolysates in small amounts, if at all, growth in such cases is dependent only on the adenine and guanine present. By means of an independent method of analysis for guanine, such as is provided by the use of the phenol reagent (9), the adenine values may be obtained as the difference between the adenine plus guanine value given by the mold and the guanine value found colorimetrically, provided the ratio of guanine to adenine is 1 or less. For ratios of from 1 to 2 moles of guanine per mole of adenine, the adenine value is one-half the total given directly by the mold. The method to be used in a particular case can easily be determined by comparison of the independent value for guanine (converted to adenine equivalents) with the value for both purines as given by assay when the standard curve based on adenine is used.

#### SUMMARY

At molar ratios to adenine of 1 or less, guanine, guanosine, and guanylic acid were utilized as efficiently for the growth of *Neurospora* mutant 28610 as were equivalent amounts of adenine. No significant increase in the amount of growth was found with higher molar ratios than 1 in the case of guanosine and guanylic acid, but when 2.5 times as much guanine as adenine were present, the mycelial weight corresponded to about 2.5 times the adenine value. Guanosine in the presence of adenosine gave results essentially similar to those found with guanine and adenine.

Xanthine in a ratio to adenine of 0.25 and to hypoxanthine of 0.5 gave the same increases in growth as would have been obtained by equivalent

amounts of adenine or of hypoxanthine. The maximum effect in the former case corresponded to about 1.25 times the adenine value and in the latter to about 1.5 times that for hypoxanthine alone.

Guanine was nearly as efficiently utilized in the presence of hypoxanthine as with adenine when the guanine-hypoxanthine ratio was 0.7 or less. As the ratio was increased to 1, however, a decrease in growth response occurred, and at ratios higher than 1.3 growth was strikingly inhibited.

Possible explanations for some of the observed effects are discussed. It is suggested that this strain of *Neurospora* is deficient in the ability to synthesize guanine as well as adenine from simple constituents and that, when growth takes place in the absence of guanine, adenine is in part converted to guanine for nucleic acid synthesis.

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# TRYPTOPHAN AND NICOTINIC ACID STUDIES IN MAN\*

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(Received for publication, July 9, 1948)

Recent studies have shown that tryptophan is effective in preventing or curing nicotinic acid deficiency in the rat (1), chick (2), mouse (3), dog (4), pig (5), and rabbit (6). Increased urinary excretion of nicotinic acid or N<sup>1</sup>-methylnicotinamide (N<sup>1</sup>-Me) after ingestion of tryptophan has been demonstrated in the rat (7), cotton rat (8), horse (8), pig (5), dog (4), calf (9), and human (10, 11).

The present experiments extend the observation (10) that the administration of tryptophan to humans is followed by an increased excretion of N<sup>1</sup>-Me. Diets which have been associated with the development of nicotinic acid deficiency in the dog and rat, and of pellagra in man, have usually contained corn products, or other sources of protein relatively low in tryptophan. In this study subjects were maintained on corn or wheat diets, which were low in total protein and B vitamins and differed mainly in tryptophan content. Tryptophan supplements were given at various times during the experimental periods. The urines of the subjects were analyzed for tryptophan-like compounds, nicotinic acid derivatives, and other B vitamins, as well as for nitrogen and creatinine. The effect of the supplements of tryptophan on tissue stores of nicotinic acid was also studied.

## EXPERIMENTAL

The subjects, with one exception (a well nourished normal male), were ward patients who were found to be free of complicating organic disease, as determined by clinical and laboratory examinations. They were transferred to a separate metabolism ward where they were permitted to be ambulatory. Periodic clinical examinations and blood studies were carried out. No significant changes in clinical status, blood count, or concentration of serum proteins were observed during the course of the experiments. The subjects were closely supervised in regard to complete urine collection and strict adherence to the diets which are shown in Table I. Variations in food intake, which were necessary in order to adjust the caloric intake of some of the subjects, were recorded. The wheat diet

\* This work was aided by grants from the Nutrition Foundation, Inc., the Williams-Waterman Fund of the Research Corporation, and the United States Public Health Service.

provided 248 gm. of unenriched white flour and cream of wheat. When the diet contained 190 gm. of unenriched degerminated corn-meal and grits and 68 gm. of wheat products, it was designated the corn diet. The cereals supplied approximately one-third of the total calories and one-half of the

TABLE I  
*Experimental Diets\**

Wheat diet			Corn diet			
	Cooked weight	Flour or cereal		Cooked weight	Flour	Corn-meal or grits
	gm.	gm.		gm.	gm.	gm.
White bread	175	123	White bread	50	35	
Biscuits	125	75	Corn bread	260	33	100
Cream of wheat	250	50	Grits	250		50
			Corn-meal mush	200		40
Total . . . . .		248			68	190
						gm.
Corned beef or salt pork . . . . .						30
Black eyed peas . . . . .						84
Turnip or mustard greens . . . . .						30
Other vegetables (onions, beets, carrots, string beans) . . . . .						250
Pineapple or apple juice . . . . .						100
Canned fruit (pears, peaches, apple sauce, fruit cocktail) . . . . .						150
Oleomargarine . . . . .						90
Marmalade or jelly . . . . .						25
Sugar . . . . .						10
Ginger ale . . . . .						180
Coca-cola . . . . .						180

The unenriched white flour was generously provided by General Mills, Inc., through the courtesy of Dr. F. C. Hildebrand and Dr. H. S. Faulkner. We are indebted to Dr. F. N. Peters of the Quaker Oats Company for the corn products used in these diets.

\* The meats, vegetables, and fruits were alternated so that the subject received the same total foods in each 2 day period.

protein. Only 10 per cent of the total protein was of animal origin. The average nutrient content (calculated) of the diets is shown in Table II. The corn diet provided approximately 150 mg. (30 per cent) less tryptophan than did the wheat diet. This is the most marked difference in the nutritive value of the two diets. After suitable control periods on these diets, DL-tryptophan<sup>1</sup> was added as a supplement. This was administered as a suspension in fruit juice with dinner and supper.

<sup>1</sup> The DL-tryptophan and the tablets containing the test dose vitamins used in these studies were generously supplied by the Winthrop-Stearns Chemical Company, Inc., through the courtesy of Mr. Kenneth Smoot.

24 hour urines were collected in amber bottles containing 15 ml. of glacial acetic acid, stored in the refrigerator, and pooled in 48 hour periods for analysis of thiamine, nicotinic acid, N<sup>1</sup>-Me, riboflavin, 4-pyridoxic acid, creatinine, and nitrogen. At the end of the experimental periods, oral test doses of 5 mg. each of riboflavin and thiamine and 50 mg. of nicotinamide were given to obtain some indication of tissue stores of these vitamins.

Thiamine was measured by a modification of the thiochrome method with a sulfite blank (12). N<sup>1</sup>-Me and 4-pyridoxic acid (as the lactone) were also measured fluorometrically by modifications of the methods of Huff and Perlzweig (13, 14). Following the administration of large amounts of DL-tryptophan, urinary products were found which interfered with fluorometric assay of 4-pyridoxic acid (14) and of riboflavin (15). The early analyses made by these methods had to be discarded.

TABLE II  
*Food Values of Experimental Diets (Calculated)*

	Corn diet	Wheat diet
Calories . . . . .	2470	2400
Protein, gm. . . . .	38	43
Tryptophan, mg. . . . .	280	430
Thiamine, mg. . . . .	0.79	0.62
Riboflavin, mg. . . . .	0.55	0.49
Nicotinic acid, mg. . . . .	5.1 (5.5)*	5.7 (6.0)*

\* Determined by analysis.

In the microbiological methods used for the determination of riboflavin, nicotinic acid, and tryptophan, 40 gm. each of glucose and of sodium acetate were used per liter of medium. *Lactobacillus casei* was used for riboflavin assay with the above change in the medium of Snell and Strong (16). *Lactobacillus arabinosus* was the test organism for assay of nicotinic acid and tryptophan with the medium of Krehl, Strong, and Elvehjem (17). When used for tryptophan analysis, 400  $\gamma$  of nicotinic acid per liter replaced the tryptophan of the original medium. The values obtained are expressed in terms of L-tryptophan.

Singal *et al.* (18) observed that in rats which had received tryptophan the apparent nicotinic acid content of the urine was markedly increased by autoclaving with strong acid. For analysis of this metabolite in human urine some of the samples were autoclaved with 1 N sulfuric acid for 1 hour at 15 pounds pressure before microbiological analysis for nicotinic acid.

In some of the experiments, tryptophan analyses were made after extraction of the urine with ether to remove compounds other than tryptophan, which had tryptophan-like activity for *Lactobacillus arabinosus* (19-21).

The pH of the urine was adjusted to 3.5 to 4 and the samples extracted twice in a separatory funnel with equal volumes of ether.

All microbiological tests were incubated at 37° for about 65 hours and titrated with 0.1 N NaOH with brom-thymol blue as indicator.

Aliquots of the urines were digested with sulfuric acid and 30 per cent hydrogen peroxide for determination of total nitrogen with Nessler's reagent. Creatinine was measured by a simple colorimetric procedure by using an alkaline picrate reagent. Readings were made with a photo-electric colorimeter.

### *Results*

Two adult females (Subject 1, M. K., age 51, weight 45 kilos, and Subject 2, I. C., age 21, weight 45 kilos) were maintained on the wheat diet for 6 days, followed by successive 18 day periods on the corn and wheat diets (Table III). During the last 8 days of the two latter periods the subjects received 5 gm. of DL-tryptophan daily (2.5 gm. with dinner and supper). The daily urinary excretion of creatinine, nitrogen, thiamine, nicotinic acid, N<sup>1</sup>-Me, and tryptophan by these subjects is shown in Table III. The creatinine figures are an approximate indication of the completeness of urine collection. In both subjects the excretion of nitrogen was lower on the corn than on the wheat diet. With tryptophan added, the average nitrogen excretion was increased 0.7 and 1.2 gm. in the two subjects on the corn diet and only 0.4 and 0.2 gm. on the wheat diet. The D-tryptophan (2.5 gm.), which presumably would not be utilized by humans (22), could contribute 0.34 gm. of nitrogen per day to the values found during supplementation.

Thiamine excretion on these diets rapidly fell to low levels when accurate determination was not possible by fluorometric methods. The slight variations in excretion on the basal diets and after tryptophan ingestion do not appear to be significant (Table III). The metabolism of large amounts of DL-tryptophan results in the excretion of substances which may interfere with the fluorometric measurement of thiamine as they do in the assay of riboflavin and 4-pyridoxic acid.

Nicotinic acid excretion, as measured microbiologically on diluted urine (not hydrolyzed), was not significantly altered by the change from the wheat to the corn diet or by administration of tryptophan. Experiments presented later (Subjects 5 and 6) show that, after tryptophan administration, urinary nicotinic acid values are markedly increased by preliminary acid hydrolysis.

N<sup>1</sup>-Me excretion was significantly decreased by changing from the initial wheat diet to the corn diet, and was greatly increased by the addition of 5 gm. of DL-tryptophan to either diet. The gradual increase in N<sup>1</sup>-Me

excretion following tryptophan administration resembles the excretion obtained with successive daily doses of nicotinamide (23). With 5 gm. of DL-tryptophan, the N<sup>1</sup>-Me excretion reached a value 6 to 12 mg. above the basal level. This increase in N<sup>1</sup>-Me, as measured by fluorometric analysis, was also demonstrated by chemical determination of total N<sup>1</sup>-methyl

TABLE III

*Effect of Tryptophan Administration on Daily Excretion of Nicotinic Acid Derivatives and Related Compounds by Subjects 1 and 2\**

Diets	Days	Creatinine		Nitrogen		Thiamine		Nicotinic acid		N <sup>1</sup> -Me		Tryptophan	
		Sub- ject 1	Sub- ject 2	Sub- ject 1	Sub- ject 2	Sub- ject 1	Sub- ject 2	Sub- ject 1	Sub- ject 2	Sub- ject 1	Sub- ject 2	Sub- ject 1	Sub- ject 2
		gm.	gm.	gm.	gm.	γ	γ	mg.	mg.	mg.	mg.	mg.	mg.
Wheat	1-2	0.65	0.99	3.7	6.6	30	63	0.4	0.6	1.1	2.2	14	9
	3-4	0.66	0.79	3.6	4.4	21	63	0.5	0.5	1.2	1.9	15	8
	5-6	0.68	0.88	4.0	4.6	26	26	0.7	0.6	1.1	1.8	15	9
Corn	7-8	0.62	0.69	2.9	3.2	10	12	0.8	0.6	0.4	1.0	7	6
	9-10	0.67	0.81	3.2	3.7	8	16	0.6	0.5	0.6	1.1	5	6
	11-12	0.64	0.87	3.4	4.4	10	10	0.7	0.6	0.6	1.0	8	7
	13-14	0.57	0.79	3.0	4.3	19	20	0.8	1.1	0.7	1.1	9	5
	15-16	0.67	0.68	3.0	3.5	12	19	0.7	0.7	0.8	1.1	7	5
Corn + 5 gm. DL-tryptophan per day	17-18	0.58	0.88	3.4	4.6	29	25	0.5	0.8	1.5	4.0	94	72
	19-20	0.67	0.81	4.2	5.2	32	29	0.6	0.7	5.3	8.5	126	79
	21-22	0.65	0.86	3.9	4.8	120?	26	0.5	0.7	7.0	9.9	115	94
	23-24	0.68	0.94	3.8	5.4	9	25	0.6	0.7	6.6	13.3	121	94
	25-26	0.68	0.88	4.0	4.5	17	17	0.7	0.8	4.5	6.7	16	8
Wheat	27-28	0.64	0.91	4.1	4.7	15	15	0.7	0.8	2.2	4.4	17	9
	29-30	0.69	0.93	4.4	5.7	15	15	0.4	0.6	2.0	3.4	18	8
	31-32	0.68	0.86	4.4	6.1	9	15	0.6	0.7	1.7	3.1	16	9
	33-34	0.72	0.91	4.3	5.3	26	32	0.7	0.8	1.4	2.5	16	9
	35-36	0.63	0.91	4.2	5.6	12	12	0.5	0.7	2.3	6.6	103	82
Wheat + 5 gm. DL-tryptophan per day	37-38	0.70	0.92	4.9	5.3	13	16	0.7	0.7	7.5	10.1	121	93
	39-40	0.69	0.87	4.7	5.2	24	18	0.7	0.6	10.7	13.5	116	91
	41-42	0.70	0.86	4.9	5.4	22	9	0.6	0.6	11.3	13.8	124	101

\* Average intake: Subject 1, wheat diet, 2300 calories, 42 gm. of protein; corn diet, 2400 calories, 37.5 gm. of protein. Subject 2, wheat diet, 2400 calories, 43 gm. of protein; corn diet, 2470 calories, 38 gm. of protein.

derivatives of nicotinic acid (24). The two methods showed similar increments in the excretion of N<sup>1</sup>-Me after tryptophan administration (10).

Tryptophan excretion on the corn diet was also significantly lower than that on the wheat diet. Previous studies have shown that tryptophan excretion may be decreased when the diet is low in this amino acid (25). After ingestion of the DL-tryptophan supplements, the excretion as measured by *Lactobacillus arabinosus* was increased to about 100 mg. Subse-

quent studies have shown that a portion of this material is not tryptophan, since ether extraction of the urine reduces the values. *Lactobacillus arabinosus* cannot utilize D-tryptophan for growth, but can use various indole derivatives to a limited extent (19-21).

The data in Table IV represent the results of a similar experiment on Subject 3 (L. B.), a 60 year old male weighing 65 kilos. The wheat diet was supplemented with tryptophan before changing to the corn diet. The findings are similar to those shown for the first two subjects. On both diets the ingestion of 5 gm. of DL-tryptophan led to an increase in the excre-

TABLE IV

*Effect of Tryptophan Administration on Daily Excretion of Nicotinic Acid Derivatives and Related Compounds by Subject 3*

Diets	Days	Creat- inine	Nitro- gen	Thia- mine	Ribo- flavin	Nicotin- ic acid	N <sup>1</sup> -Me	Trypto- phan
		gm.	gm.	$\gamma$	$\gamma$	mg.	mg.	gm.
Wheat (2370 calories, 41 gm. protein)	1- 2	1.1	6.2	60	228	0.9	1.8	13
	3- 4	1.2	5.7	45	224	0.8	1.7	14
	5- 6	1.1	5.9	25	115	0.7	1.3	15
	7- 8	1.1	6.2	34	83	0.7	1.7	14
	9-10	1.1	5.3	32	76	0.9	2.0	12
Wheat + 5 gm. DL-tryptophan per day	11-12	1.0	5.4	23	68	0.7	1.9	88
	13-14	1.1	6.2	30	70	0.8	4.5	110
	15-16	1.0	5.9	19	65	1.0	6.5	114
	17-18	1.0	5.8	21	60	0.8	8.5	107
	19-20	1.1	5.4	23	59	0.7	6.2	16
Corn (2450 calories, 37 gm. protein)	21-22	1.1	5.6	27	62	0.7	4.4	12
	23-24	1.1	5.1	21	47	0.7	3.3	12
	25-26	1.0	5.2	34	53	0.6	3.2	12
	27-28	1.0	4.9	38	49	0.5	2.9	12
	29-30	1.0	5.6	120?	51	0.6	6.0	107
Corn + 5 gm. DL-tryptophan per day	31-32	1.0	5.6	17	52	0.7	6.9	111
	33-34	1.1	6.3	27	55	0.8	9.2	112
	35-36	1.0	6.0	15	53	0.7	8.6	117

tion of N<sup>1</sup>-Me of about 6 mg. In the second period of supplementation the rise in excretion of N<sup>1</sup>-Me was more rapid than in the first. This excretion of extra N<sup>1</sup>-Me persisted for several days after the supplementation was discontinued, whereas the excretion of tryptophan itself returned to the basal level much more rapidly. These effects were also observed in the first two subjects.

The thiamine excretion of this subject was similar in its variations to that found in Subjects 1 and 2. Urinary riboflavin, which was measured microbiologically, decreased rapidly for a few days, and then slowly reached a plateau of about 50  $\gamma$  per day (Table IV). Riboflavin excretion was not

significantly affected by the change from the wheat to the corn diet or by the addition of tryptophan to either diet.

The nitrogen excretion of this male subject (Table IV) was higher than that found in the two females (Table III) on the same diets. There was virtually no change in nitrogen excretion when tryptophan was added to the wheat diet, while there was an increase of about 0.7 gm. of nitrogen per day when the corn diet was supplemented with tryptophan.

The effect of the addition of 5 gm. of DL-tryptophan to a good normal diet was determined in a well nourished male (Subject 4, H. S., age 31, weight 75 kilos). The diet, which was not controlled as rigorously as in the previous experiments, provided about 100 gm. of protein per day as well as adequate amounts of all of the vitamins. In a 3 day basal period

TABLE V  
*Effect of Tryptophan Administration on Daily Excretion of Nicotinic Acid Derivatives and Related Compounds by Subject 4*

Diets	Days	Creat- inine	Nitro- gen	Thia- mine	Ribo- flavin	Nicotin- ic acid	N <sup>1</sup> -Me	Trypto- phan
		gm.	gm.	γ	mg.	mg.	mg.	mg.
Normal control	1	2.1	14.0	210	1.6	1.0	7.5	15
	2	2.1	14.3	160	1.1	0.8	8.1	15
	3	2.1	13.5	115	1.1	0.7	7.2	15
Normal control + 5 gm. DL- tryptophan per day	4	2.0	13.4	180	0.9	0.8	10.7	78
	5	1.9	13.3	180	1.2	1.0	14.2	78
	6	2.2	13.9	215	1.1	1.0	17.5	90
	7	2.1	12.5	165	1.0	0.9	18.0	89
	8	2.1	12.3	170	1.1	1.0	18.3	79

this subject excreted about 14 gm. of nitrogen, 15 mg. of tryptophan, and 7.5 mg. of N<sup>1</sup>-Me per day (Table V). The ingestion of 5 gm. of DL-tryptophan daily for 5 days led to a 10 mg. increase in excretion of N<sup>1</sup>-Me. About 90 mg. of tryptophan compounds were excreted. This is slightly less than that found in the other three subjects on poorer diets. Nitrogen, thiamine, and riboflavin excretion were not significantly affected by tryptophan administration.

Since the first four subjects excreted 6 to 12 mg. of N<sup>1</sup>-Me above the basal output when 5 gm. of DL-tryptophan were administered daily, two experiments were set up with other levels of tryptophan to determine whether there is a quantitative relationship between the amount of tryptophan added to the diet and the extra N<sup>1</sup>-Me excreted. These experiments were carried out in persons maintained on the wheat diet. Subject 5 (C. N.), a 63 year old woman weighing 46 kilos, was given this diet for 30 days. During the second and third 10 day periods she received 2 and 5 gm.

of DL-tryptophan daily, respectively (Table VI). The increase in N<sup>1</sup>-Me excretion after ingestion of tryptophan was lower than that found in the other subjects on the wheat diet. When 2 gm. of DL-tryptophan were administered, there was an increase in excretion of N<sup>1</sup>-Me of 0.5 to 2.7 mg. per day above the basal level of 0.8 mg. Addition of 5 gm. of DL-tryptophan to the diet led to an increase of 3.9 to 6.5 mg. in N<sup>1</sup>-Me excretion. The average increase after 2 gm. of tryptophan, 1.7 mg., is approximately 30 per cent of the average rise after 5 gm. of tryptophan, 5.7 mg. The

TABLE VI  
*Effect of Administration of Varying Levels of Tryptophan on Daily Excretion of Nicotinic Acid and Tryptophan Compounds by Subject 5*

Diets	Days	Nicotinic acid		N <sup>1</sup> -Me	Tryptophan	
		Before hydrolysis	After hydrolysis		Before ether extraction	After ether extraction
		mg.	mg.	mg.	mg.	mg.
Wheat (2400 calories, 40 gm. protein)	1- 2	0.3	0.4	0.6	5.4	4.7
	3- 4	0.3	0.8	1.0	5.4	
	5- 6	0.5	1.1	1.0	5.0	4.0
	7- 8	0.4	1.3	0.7	5.2	4.5
	9-10	0.4	1.1	0.7	5.5	4.3
Wheat + 2 gm. DL-tryptophan per day	11-12	0.4	1.7	1.3	12	
	13-14	0.4	1.6	2.5	20	11
	15-16	0.5	1.7	2.6	20	
	17-18	0.5	1.6	2.5	16	8
	19	0.4	2.1	3.5	34	15
Wheat + 5 gm. DL-tryptophan per day	22	0.4	2.5	7.3	42	
	23-24	0.3	1.8	4.7	34	12
	25-26	0.4	3.4	6.6	48	19
	27-28	0.5	2.5	6.5	37	17
	30	0.4	2.8	7.3	40	17

extra excretion of N<sup>1</sup>-Me toward the end of the 10 day period when 2 gm. of tryptophan were given was 2.7 mg. This appears to be a better indication of the conversion of tryptophan to N<sup>1</sup>-Me than does the average value. The extra excretion of N<sup>1</sup>-Me on this basis is approximately the theoretical 40 per cent of that found after administration of 5 gm. of tryptophan.

In this experiment it was also shown (Table VI) that autoclaving the urines for 1 hour with 1 N sulfuric acid markedly increased the nicotinic acid values in specimens obtained after tryptophan ingestion. Singal *et al.* (18) obtained similar findings when rat urines were autoclaved for 15 minutes. With human urine the values are further increased by longer hydrolysis (up to 4 hours). 1 hour hydrolysis was chosen as a convenient time to show this effect of tryptophan administration.



The excretion of tryptophan-like compounds by Subject 5 (Table VI) is lower than that found in the previous subjects, both before and after supplementation. Several of the urines in this experiment were analyzed for tryptophan after two extractions (at pH 4) with equal volumes of ether. This procedure does not remove tryptophan but does remove other compounds with tryptophan-like activity for *Lactobacillus arabinosus* (20, 21). On the basal diet, the ether extraction reduced the tryptophan value by about 1 mg. per day, but after administration of tryptophan the extraction removed about one-half of the tryptophan activity.

Thiamine and riboflavin excretion of this subject (not shown in Table VI) decreased during the course of the experiment and was not affected by tryptophan administration.

Subject 6 (W. H.), a 30 year old male weighing 65 kilos, was given DL-tryptophan in amounts of 2, 5, and 10 gm. per day while on the wheat diet, to test further the proportionality between tryptophan administration and N<sup>1</sup>-Me excretion (Table VII). Since the normal metabolism of tryptophan, including its conversion to N<sup>1</sup>-Me, is known to require pyridoxine compounds (26-30), pyridoxal or pyridoxine was given with the tryptophan to see whether this affected the amount of conversion of tryptophan to nicotinic acid-like compounds.

In Subject 6, the administration of 2 gm. of DL-tryptophan per day for 10 days led to an increased excretion of 1 to 6.5 mg. of N<sup>1</sup>-Me (average 3.1 mg.) above the average basal value of 2.1 mg. The values obtained toward the end of the initial tryptophan period again appear to be a better indication of the amount of possible conversion of tryptophan to N<sup>1</sup>-Me than does the average value. Days 22 to 27 (Table VII) represent 6 of the 8 days during which this subject received 5 gm. of DL-tryptophan daily. The average excretion of N<sup>1</sup>-Me was 14.7 mg. more than that of the basal period. During Days 39 to 48 the subject received 10 gm. of tryptophan per day and excreted about 23 mg. more N<sup>1</sup>-Me than when on the control diet. It will be noted that the extra excretion of N<sup>1</sup>-Me during these 10 days decreased from an initial value of 27 mg. to about 20 mg. However, the above data show that the additional N<sup>1</sup>-Me excreted at each of the three levels of intake of tryptophan was roughly proportional to the amount administered. Table VII also shows a progressive increase in excretion of acid-hydrolyzable precursors of nicotinic acid as the amount of tryptophan administered was increased.

The addition of 20 mg. of pyridoxal hydrochloride<sup>2</sup> to the 5 gm. of tryptophan supplement (Days 29 to 34) was accompanied by a slight decrease in excretion of N<sup>1</sup>-Me. The removal of this pyridoxal from the diet led to no further change in N<sup>1</sup>-Me excretion. When 10 gm. of tryptophan were

<sup>2</sup> The pyridoxal hydrochloride was kindly furnished by Merck and Company, Inc.

administered (Days 39 to 62), the excretion of N<sup>1</sup>-Me started at 29 mg. per day and decreased before any pyridoxine compound was added. The

TABLE VII  
*Effect of Administration of Varying Levels of Tryptophan and of Pyridoxine Compounds on Daily Excretion of Nicotinic Acid and Tryptophan Compounds by Subject 6*

Diets	Days	Nicotinic acid		N <sup>1</sup> -Me	Tryptophan	
		Before hydrolysis	After hydrolysis		Before ether extraction	After ether extraction
		mg.	mg.	mg.	mg.	mg.
Wheat (2580 calories, 45 gm. protein)	1- 2	0.5	1.3	1.3	17.0	17.0
	3- 4	0.9	2.1	2.2	17.5	
	5- 6	0.7	2.0	2.1	19.8	19.6
	7- 8	0.6	1.6	2.3	17.6	17.0
	9-10	0.5	1.7	2.6	19.4	18.0
Wheat + 2 gm. DL-tryptophan per day	11-12	0.8	2.3	3.1	47	31
	13-14	0.7	2.5	4.7	42	
	15-16	1.3	3.3	5.6	46	39
	17-18	0.8	2.3	4.2	43	34
	19	1.1	3.2	8.5	50	39
Wheat + 5 gm. DL-tryptophan per day	22	0.7	3.1	15	98	62
	23-24	0.9	3.7	19	110	74
	25-26	0.7	3.6	15	110	71
	27	0.8	3.2	19	90	65
	29-30	0.9	4.0	16	112	82
Wheat + 5 gm. DL-tryptophan + 20 mg. pyridoxal HCl per day	31-32	0.8	3.2	14	108	77
	33-34	0.6	2.9	11	98	65
Wheat + 5 gm. DL-tryptophan per day	35-36	0.8	2.9	11	102	64
	37-38	0.7	3.1	10	97	58
Wheat + 10 gm. DL-tryptophan per day	39-40	1.0	5.8	29	167	98
	41-42	0.9	5.7	26	169	91
	43-44	0.9	7.6	24	187	113
	45-46	0.9	7.2	22	172	109
	47-48	1.0	6.7	23	188	116
Wheat + 10 gm. DL-tryptophan + 20 mg. pyridoxine HCl per day	49-50					
	51-52	1.0	7.9	23	194	115
	53-54	0.8	7.2	17	194	120
	55-56	1.0	8.8	21	192	127
Wheat + 10 gm. DL-tryptophan per day	57-58	0.8	6.5	17	165	93
	59-60	0.8	6.9	15	174	97
	61-62	0.8	7.7	18	196	121

administration of 20 mg. of pyridoxine hydrochloride with the tryptophan led to no significant change in N<sup>1</sup>-Me excretion, although a slight decrease did occur when the pyridoxine was removed. From these data, it appears that continued administration of large amounts of tryptophan leads to a

decrease in  $N^1$ -Me excretion after the initial peak is reached. Whether the pyridoxine compounds had any effect upon this decrease is questionable. Other experiments in this laboratory<sup>3</sup> with smaller tryptophan supplements indicate that extra pyridoxine either has no effect or leads to a slight decrease in excretion of  $N^1$ -Me. Experiments with rats (27–29) showed a decrease in the conversion of tryptophan to  $N^1$ -Me on pyridoxine-deficient diets. No animal experiments with various levels of pyridoxine in the diet have been reported.

Excretion of tryptophan by Subject 6 (Table VII) was similar to that found in the other subjects. Extraction of the urine with ether again showed the presence of tryptophan-like metabolites, which increased when additional tryptophan was administered.

The 4-pyridoxic acid excreted by several of these subjects was measured fluorometrically (14). The values obtained during the periods of tryptophan administration were 0 to 5 mg. higher than the basal levels of 1 to 4 mg. per day. Recent experiments have shown that the ether extraction, which removes compounds with tryptophan-like activity, reduces the fluorometric values of 4-pyridoxic acid to approximately basal values.<sup>4</sup> 4-Pyridoxic acid is not removed from urine by this procedure. It therefore appears likely that large amounts of tryptophan metabolites interfere with the fluorometric assay of 4-pyridoxic acid.

*Tissue Saturation*—The data on urinary excretion (Tables III to VII) indicate that tryptophan is converted to nicotinic acid compounds by man. Part of this nicotinic acid should be retained to maintain or increase body stores of nicotinic acid. Tissue saturation of nicotinic acid was compared with that of thiamine and riboflavin by means of urinary excretion tests after an oral dose of these vitamins. A tablet containing 5 mg. of thiamine, 5 mg. of riboflavin, and 50 mg. of nicotinamide was administered to the subjects the morning after the completion of the above experiments, and the 4 hour urinary excretion of thiamine, riboflavin, and  $N^1$ -Me was measured. In three of the subjects this test was also performed a few days prior to the start of the experiment. The findings are presented in Table VIII and may be compared with those obtained in control subjects on adequate diets.

Subject 4 was maintained on a good diet throughout the experiment and after 5 days of tryptophan administration showed no significant change in test dose excretion of thiamine or riboflavin, whereas the  $N^1$ -Me excretion was markedly increased. The corn and wheat diets of the other five subjects were low in thiamine, riboflavin, and nicotinic acid (Table II). After 30 to 62 days on these diets, the excretion following a test dose showed

<sup>3</sup> Sarett, H. P., and Goldsmith, G. A., unpublished data.

<sup>4</sup> Sarett, H. P., Haas, C. C., and Loeb, J. M., unpublished data.

evidence of mild to severe depletion of thiamine and riboflavin in four of these subjects. However, the excretion of N<sup>1</sup>-Me indicated fair to good body stores of nicotinic acid.

In Subjects 5 and 6 comparison can be made of the values obtained before and after the experimental period. In Subject 5 the excretion of N<sup>1</sup>-Me was essentially unchanged, whereas there was a sharp decrease in thiamine and riboflavin excretion. Subject 6 also showed a diminished output of riboflavin and thiamine in the second test, while the excretion of N<sup>1</sup>-Me was greater at this time. The addition of tryptophan to a diet low in nicotinic acid appears, therefore, to maintain or increase body stores of nicotinic acid.

TABLE VIII

*Urinary Excretion (4 Hour) of Thiamine, Riboflavin, and N<sup>1</sup>-Methylnicotinamide by Subjects after Oral Test Dose of 5 Mg. of Thiamine, 5 Mg. of Riboflavin, and 50 Mg. of Nicotinamide*

Subject	Thiamine	Riboflavin	N <sup>1</sup> -Methyl-nicotinamide	Remarks
	$\gamma$	mg.	mg.	
Normals (12)	211 (82-376)	1.9 (1.3-2.4)	7.5 (5.0-13.1)	
1. M. K.	35	1.3	9.5	After experiment, Table III
2. I. C.	184	1.6	12.5	" " " III
3. L. B.	21	1.2	6.5	" " " IV
4. H. S.	167	2.1	9.3	Control
4. H. S.	224	1.9	14.8	After experiment, Table V
5. C. N.	169	1.7	3.4	Control
5. C. N.	7	0.5	3.7	After experiment, Table VI
6. W. H.	54	1.8	4.0	Control
6. W. H.	26	0.1	8.0	After experiment, Table VII

## DISCUSSION

The excretion of N<sup>1</sup>-Me by humans appears to be related to the nicotinic acid and tryptophan content of the diet. When tryptophan is administered, the extra excretion of nicotinic acid compounds is approximately proportional to the amount of tryptophan added to the diet. This suggests that a definite portion of *extra* tryptophan is converted to nicotinic acid. Several workers (18, 29, 31, 32) have found that extra casein in the rat diet leads either to no increase in excretion of N<sup>1</sup>-Me or to an increase less than that which would be expected if free tryptophan was administered in amounts equivalent to that present in the casein. It appears, therefore, that, although extra free tryptophan in the diet leads to the formation of nicotinic acid or N<sup>1</sup>-Me, the ingestion of this tryptophan in protein may lead to its utilization along other pathways. Some of the tryptophan may be converted to nicotinic acid, which might be required in the deposition

and utilization of the added protein (33). Further elucidation of this subject may be obtained when assay of the 6-pyridone of N<sup>1</sup>-methylnicotinamide (34) is feasible.

The higher excretion of N<sup>1</sup>-Me by subjects on the basal wheat diet in contrast to the corn diet may be due to the larger amount of tryptophan in wheat diet. Both diets provide about the same amount of nicotinic acid and differ little in total content of protein. It is possible that a "pellagra-genic" substance in corn (35) increases the nicotinic acid or tryptophan requirement. This substance may act by inhibition of the conversion of tryptophan to nicotinic acid or of the utilization of nicotinic acid. However, the presence of this substance is not necessary to explain the difference between the corn and the wheat diets in view of their relative content of tryptophan. Tryptophan-deficient proteins and acid-hydrolyzed proteins have retarded rat growth in the same manner as do corn products low in tryptophan (36, 37). The addition of specific amino acids to diets low in protein and nicotinic acid also causes an imbalance which depresses rat or chick growth and is reversed by nicotinic acid or tryptophan (38-40).

The metabolism of large amounts of DL-tryptophan (or L-tryptophan<sup>5</sup>) by humans leads to the excretion of two substances which may be intermediates in the formation of nicotinic acid or by-products in the metabolism of tryptophan. The first of these is a substance which has tryptophan-like activity for *Lactobacillus arabinosus* but can be extracted from acidified urine with ether. Concentration of this material and preliminary tests indicate that it is an acidic compound containing the indole nucleus.<sup>6</sup> The second substance was originally found in the urine of rats which received tryptophan (18). Autoclaving this compound in strong acid converts it to nicotinic acid or a compound with nicotinic acid activity for *Lactobacillus arabinosus*. Small amounts of the two compounds described above may appear in the urine of subjects who receive no supplementary tryptophan. Administration of nicotinic acid or nicotinamide does not lead to any increase in the levels of these compounds in the urine.<sup>6</sup>

#### SUMMARY

The urinary excretion of creatinine, nitrogen, thiamine, tryptophan, riboflavin, nicotinic acid, and N<sup>1</sup>-methylnicotinamide (N<sup>1</sup>-Me) was measured in human subjects on corn and wheat diets. The excretion of N<sup>1</sup>-Me and of tryptophan was higher on the wheat than on the corn diet. This may be due to the difference in the tryptophan content of the diets.

The administration of DL-tryptophan with either diet led to an increase in excretion of N<sup>1</sup>-Me and of a substance which had nicotinic acid activity

<sup>5</sup> Sarett, H. P., unpublished data.

<sup>6</sup> Sarett, H. P., and Loeb, J. M., unpublished data.

after autoclaving with strong acid. An increase in urinary tryptophan and a related metabolite was also noted. Similar findings were obtained when tryptophan was administered to a subject on an adequate diet. The increase in N<sup>1</sup>-Me excretion was approximately proportional to the amount of tryptophan added to the diet.

Pyridoxine or pyridoxal did not appreciably affect the urinary excretion of N<sup>1</sup>-Me when tryptophan was administered.

The administration of tryptophan had no effect on the urinary excretion of thiamine and riboflavin.

Urinary excretion of N<sup>1</sup>-Me after a test dose of nicotinamide showed that the administration of tryptophan had contributed to the maintenance of body stores of nicotinic acid.

The authors wish to thank the following people for cooperation in these studies: Dr. Roy E. Butler for clinical assistance, Janis Gibbens and Winifred Bouvet for planning and supervising the diets, and Antoinette Dingraudo, Phyllis Gerhardt, Carol Haas, and Janice Loeb for their technical assistance.

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# THE REACTION OF FORMALDEHYDE WITH PROTEINS

## VII. DEMONSTRATION OF INTERMOLECULAR CROSS-LINKING BY MEANS OF OSMOTIC PRESSURE MEASUREMENTS

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(Received for publication, September 7, 1948)

It was suggested by Meyer (1) and again by Künzel (2) that the tanning and hardening effect of formaldehyde on proteins might be ascribed to the formation of methylene cross-links. The work of Gustavson (3) and Nitschmann and Hadorn (4) included experimental evidence and indicated that free amino groups were essential for the cross-linking. Preceding papers from this Laboratory (5, 6) have shown that at room temperature, and within the range of pH 3 to 9, methylene cross-links can be formed between amino groups on the one hand and amide, guanidyl, indole, phenol, or imidazole groups on the other. No evidence has yet been obtained that, under these mild conditions, cross-linkage occurs between two amino groups or between amino and peptide groups, possibilities that have at various times been suggested (2-4).

Evidence for the involvement of the various groups in cross-linking was obtained primarily through study of the behavior of model systems including, besides formaldehyde, either two low molecular weight compounds or one protein or polymer and one low molecular weight compound (5, 6). The formation of cross-links between two groups, both furnished by protein, was difficult to prove unequivocally, although evidence based upon the amounts of formaldehyde bound irreversibly by various proteins was suggestive. Thus, proteins containing appreciable amounts of amino and also phenol or imidazole groups bound considerably more formaldehyde in a manner irreversible by acid hydrolysis than did derivatives in which the amino groups had been selectively acetylated (6).

Evidence that methylene cross-linkage can be achieved does not necessarily prove that protein molecules can be linked together, since it is equally possible that the reaction might affect only pairs of reactive groups within a single molecule. However, the insolubility, resistance to swelling, etc., of formaldehyde-tanned proteins certainly suggest the formation of a tridimensionally stabilized structure (1-3). In line with this concept, it has now been possible to demonstrate intermolecular cross-linking through the marked increases in the average molecular weight of proteins after treat-

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ment with formaldehyde even under conditions which do not lead to the formation of insoluble products.<sup>1</sup>

#### EXPERIMENTAL

*Materials*—The crystalline egg albumin was obtained by the procedure of Kekwick and Cannan (7); gliadin, by an unpublished method of alcohol fractionation; polyglutamine, as previously described (8). Amino-acetylated proteins were prepared with acetic anhydride in cold sodium acetate solution (9). Crystalline bovine serum albumin and other reagents were commercial products. Ovomuroid was kindly supplied by Dr. H. Line-weaver of this Laboratory.

*Methods*—Average molecular weights were estimated by determining the osmotic pressure of the protein derivatives in buffered solutions containing 7 to 10 M urea. In this manner the possibility of molecular aggregation through secondary or coordinate bonds was minimized. The apparatus and technique described by Bull (10, 11) were used except that the osmometers were equipped with ground glass joints as suggested by Dr. H. S. Owens of this Laboratory. In urea solutions attainment of equilibrium occasionally required runs of 10 days. No attempt was made to reach equilibrium more rapidly by adjustment of the sample level during runs. All measurements were made at 24.9° (water bath).

Sample concentrations, which were 2.2 per cent or less in all cases, were determined by dialysis of an aliquot to remove urea, dilution of the aliquot to a definite volume, and estimation of nitrogen content by a micro-Kjeldahl procedure. The Folin phenol reagent also was used occasionally, by the method described by Anson (12), to determine sample concentrations and, applied to buffers from the osmometers, to make certain that no protein nitrogen was being lost through the membrane.

The method of calculation of apparent molecular weight was that given by Bull (11). The values so obtained have been referred to as *apparent* molecular weight, since, as Burk and Greenberg (13) and Scatchard, Batchelder, and Brown (14) among others have shown, the osmotic pressures developed by a protein sample run at various concentrations are seldom directly proportional to the sample concentration. Real solutions give osmotic pressures higher than those predicted by the van't Hoff theory; extrapolation of the pressure-concentration ratio to zero concentration is necessary in order to determine the true molecular weight of the solute. In general, the value calculated from a single osmotic pressure determination for the apparent molecular weight of a protein thus will be lower than the true molecular weight. The difference will decrease as the sample concentration is decreased, as the pH of the solution approaches the isoelectric

<sup>1</sup> Presumably the insoluble products had even higher molecular weights, but simple methods for measuring their magnitude do not seem to be available.

pH of the sample, and as the ionic strength of the solvent is increased. The true molecular weight of many of the samples reported in Tables I to V thus may have been appreciably higher than the value given.

The first osmotic pressure determinations were performed in buffered solutions containing no urea. Under such conditions the molecular weights of untreated proteins were in good agreement with values of the literature, and formaldehyde was found to have caused manifold increases above these values. When the same samples were analyzed in 6.7 to 10 M urea, similar values were obtained for all proteins and derivatives studied, except for untreated egg albumin. This protein when analyzed in urea solution gave inconsistent molecular weights, varying around 75,000, while in the absence of urea a value of 45,000 was obtained, in agreement with that reported by Bull (11). A possible explanation of this observation was that autoxidation of sulfhydryl groups exposed by urea denaturation might lead to intermolecular disulfide bonds and thus to progressively larger molecules.<sup>2</sup> The fact that of the proteins used only egg albumin, and, to a much less extent, serum albumin, showed this phenomenon supported the above explanation, since egg albumin was the only protein containing numerous masked —SH groups of a type to be rendered reactive by urea.

To avoid participation of —SH groups in such secondary cross-linking, iodoacetamide was added to the urea solutions of egg albumin and serum albumin. (The authors are indebted to Dr. H. B. Bull for this suggestion.) This procedure yielded consistent molecular weight values for the untreated proteins in urea solution which were somewhat lower than those of the literature (by 10 to 20 per cent), while the formaldehyde derivatives showed considerably higher values. The procedure as adopted for the albumins was as follows: 4 mg. of iodoacetamide per 100 mg. of protein were added, followed by solid urea, to give approximately a 5 M concentration. After the sample had stood overnight, it was dialyzed against several portions of urea-buffer solution, the last portion being used in the osmometer buffer chamber.<sup>3</sup>

Samples of gliadin and polyglutamine were dialyzed directly against urea-buffer solution without the addition of iodoacetamide.

Details of the conditions of formaldehyde treatment are indicated in

<sup>2</sup> The same phenomenon probably explains the results obtained by Bull and Currie with  $\beta$ -lactoglobulin (10). In 6 M urea, the apparent molecular weight was 21,700 in 7 M urea, 26,100, and in 8 M urea, greater than 30,000.

<sup>3</sup> A 3 year-old sample of crystalline egg albumin which proved to contain an appreciable amount of denatured protein showed in 10 M urea solution an apparent molecular weight of 59,000. Iodoacetamide effected in this case only a small decrease (to 55,000), but in the presence of 0.01 M thioglycol a molecular weight of 36,000 was obtained. If this "regenerated" egg albumin was dialyzed against urea to remove the thioglycol, the molecular weight rose (through autoxidation) again to 44,000, while treatment with iodoacetamide prior to dialysis gave a final molecular weight in 10 M urea of 33,000.

Tables I to V. After reaction, which was generally permitted to proceed for 10 to 14 days at room temperature, the products were isolated by dialysis for 3 days against running tap water, followed by 3 days against three successive changes of distilled water at 3°. Osmotic pressure measurements were made in duplicate on each preparation, and averaged. Many of the molecular weights given also represent averages of values obtained on two or more preparations.

### Results

*Proteins of Average Composition*—Tables I and II show the apparent molecular weights of bovine serum albumin and egg albumin after treatment with formaldehyde under various conditions. The experimental conditions had to be chosen in such a manner as to avoid the formation of insoluble products, since, in agreement with expectation, proteins rendered insoluble through formaldehyde treatment were generally not soluble even in high concentrations of urea or other disaggregating solvents and thus could not be used for osmotic pressure studies. However, the increases observed with the only partially cross-linked and still soluble preparations are sufficiently marked to prove the point that cross-linking occurs. The most crucial factor in the experimental conditions seemed to be a high protein concentration. With 10 per cent protein solutions cross-linking occurred at formaldehyde concentrations ranging from 0.4 to 10 per cent, both at pH 3.5 and 7.5. At 1 per cent protein concentration, there was no cross-linking. At room temperature, the maximal molecular weight when the protein was not made insoluble was obtained in 5 to 15 days (Table I, foot-note). At an elevated temperature (40°) an insoluble product resulted under otherwise the same conditions after 2 days, but a soluble derivative of increased molecular weight was obtained through the use of a low formaldehyde concentration. The latter reaction conditions resemble those usually employed in the preparation of vaccines and toxoids.

The most important chemical prerequisite appeared to be the presence of amino groups, as illustrated by the unsuccessful attempts to produce cross-linking under even the most favorable conditions with the amino-acetylated derivatives of bovine serum albumin and egg albumin (foot-note, Tables I and II).

As previously observed (5) cross-linking between protein groups could be prevented by the addition of small molecular weight amides or secondary amines (Table I, foot-note). Under such conditions the added compound is bound through methylene to protein groups, thus rendering these unavailable for linkage with other protein groups. The cross-linking action of added *primary* amines will be discussed below.

TABLE I  
*Apparent Molecular Weights of Soluble Preparations of Formaldehyde-Treated Bovine Serum Albumin*

Protein <i>per cent</i>	Reaction conditions*		Apparent mol. wt.†
	Concentration of formaldehyde <i>per cent</i>	Final pH	
11	4.4	3.5	330,000‡
10	5	3.5	262,000§
10	1	3.5	78,000
10	0.4	3.8	95,000¶
5	10	3.5	80,000
1	5	3.5	61,000
1	0.5	3.5	53,000
10	5	7.5	150,000
10	1	7.5	90,000
10	0.4	7.3	200,000¶
5	10	7.5	162,000
1	0.5	7.5	55,000
10	None	3.5	41,000

\* Reactions were permitted to proceed for 10 to 15 days at room temperature (22–26°) unless otherwise stated. In one experiment samples were analyzed after 1, 2, and 5 days (10 per cent protein, 5 per cent formaldehyde, pH 3.5). The apparent molecular weights were 102,000, 152,000, and 223,000, respectively. Reaction for 12 days at 3–5° gave a product with a molecular weight of 120,000, under otherwise identical conditions; at 40°, the product was insoluble after 2 days.

† Unless otherwise stated, osmotic pressure measurements were performed at pH 7.5 to 7.9 in 0.05 M phosphate buffers, in the presence of 6.7 M urea, 0.05 M NaCl, and at times iodoacetamide (see the text). Untreated bovine serum albumin showed a molecular weight of about 62,000 under these conditions with or without added iodoacetamide.

‡ Analysis performed in 0.05 M acetate-6.7 M urea, pH 5.

§ When the solution also contained 10 per cent acetamide, cross-linking between protein amino and amide groups was largely suppressed in favor of fixation of the acetamide through methylene groups to the amino groups of the protein. The apparent molecular weight of such a preparation was 61,000. Cross-linking was decreased in similar manner by the addition of proline; the molecular weight was 95,000. A primary amine, *e.g.* alanine, appeared to favor cross-linking through its amino group between pairs of protein amide groups; the molecular weight was 180,000. The amino acid concentration in these experiments was 10 per cent, the reaction period 6 days, and the final pH 3.9. A similar experiment was performed with addition of methylamine or dimethylamine (6.7 per cent protein and amine hydrochlorides, 8 per cent formaldehyde; 3 days reaction; final pH 4.6 and 4.8). The molecular weights were 134,000 and 69,000 respectively.

|| When aminoacetyl serum albumin was used under the same conditions at pH 3.5, the apparent molecular weight was 41,000 (52,000 when the formaldehyde concentration was 7.5 per cent); after reaction at pH 7.5, a molecular weight of 56,000 was obtained.

¶ These reactions were performed at 40° (13 days). Such conditions, at neutrality, resemble those of vaccine formation.

An additional water-soluble protein used in this study was ovomucoid, which has recently been identified with the trypsin inhibitor of egg white (15). This protein gave an apparent molecular weight of 26,000 (6.67 M urea, 0.05 M acetate, 0.05 M sodium chloride, pH 5.4 to 5.7), but after treatment with formaldehyde (10 per cent protein, 8 per cent HCHO, 0.8 per cent acetic acid, 5 days) the molecular weight was increased to 101,000.

*Proteins Rich in Amide Groups*—A further series of experiments was performed with gliadin. In contrast to most proteins, gliadin is rendered more water-soluble by formaldehyde treatment in all but very acid solu-

TABLE II  
*Apparent Molecular Weights of Soluble Preparations of Formaldehyde-Treated Egg Albumin*

Reaction conditions*			Apparent mol. wt.†
Protein	Concentration of formaldehyde	Final pH	
<i>per cent</i>	<i>per cent</i>		
10	5	3.5	113,000
1	5	3.5	44,000
10	10	7.5	107,000
10	5	7.5	120,000‡
10	1	7.5	104,000
1	5	7.5	40,000
1	0.5	7.5	38,000
10	None	7.5	38,000

\* The reactions were permitted to proceed for 10 to 14 days at room temperature.

† Osmotic pressure measurements were performed in 0.05 M phosphate-0.05 M NaCl-10 M urea at pH 7.8 to 8.5 and in the presence of iodoacetamide (see the text). Untreated egg albumin showed a molecular weight of about 36,000 under these conditions and in the presence of iodoacetamide.

‡ Aminoacetyl egg albumin treated with formaldehyde under the same conditions showed an apparent molecular weight of 34,000.

tions. This is probably due to the fact that the low amino nitrogen of gliadin does not favor extensive cross-linking, while the transformation of amide to amidomethylol groups may lead to increased solubility in water. When gliadin was treated in 10 per cent solution with 19 per cent formaldehyde in 50 per cent acetic acid (final pH 2.5), an approximate doubling of the molecular weight (30,000 to 59,000) was obtained,<sup>4</sup> while in more

<sup>4</sup> Below pH 2 to 3, some methylene cross-linking may occur between pairs of amide groups, as demonstrated in a model experiment by the introduction of acetamide into polyglutamine by formaldehyde treatment. The fixation of acetamide at pH 1.9 (5 days at 23°) was indicated by an amide nitrogen content of 62 per cent of the total nitrogen in the dialyzed derivative. Unchanged polyglutamine, or a sample treated with formaldehyde and acetamide at pH 3.1, had amide nitrogen contents corresponding to 48 and 47 per cent of the total N.

dilute solution (4 per cent protein, 7.5 per cent formaldehyde, 0.6 M acetic acid, final pH 3.1) the average molecular weight was increased to only about 41,000. Such reaction mixtures were suitable for the demonstration of the effect of added ammonium ions which, presumably according to reaction I ( $R = H$ ), could substitute for protein amino groups in favoring cross-linking.

TABLE III

*Apparent Molecular Weights of Soluble Preparations of Gliadin Treated with Formaldehyde in Presence or Absence of Various Amines in 0.6 M Acetic Acid Solution\**

Added compound†	Final pH	Apparent mol. wt.‡
Ammonium chloride (5).....	2.6	125,000§
“ acetate (32).....	3.4	145,000
Methylamine, HCl (5).....	2.6	58,000
Dimethylamine, HCl (5).....	2.6	49,000
Alanine (32).....	3.2	45,000
Ethylenediamine (32).....	3.9	51,000
“ 2HCl (10).....	2.5	73,000
Piperazine, 2HCl (5).....	2.6	101,000
“ “ (10).....	2.5	152,000

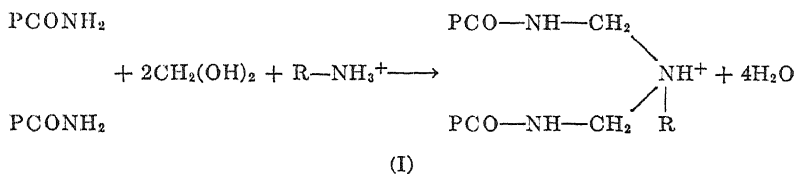
\* The concentrations of protein and formaldehyde were 4 and 7.5 per cent, respectively; the reaction time, 3 to 5 days at 23°.

† The figures in parentheses represent equivalents used per 10<sup>4</sup> gm. of protein.

‡ The osmotic pressure measurements were performed in 0.05 M phosphate-10 M urea, pH 7.7 to 8.7. The untreated gliadin showed a molecular weight of 31,000 under these conditions.

§ In a control experiment, gliadin treated with ammonium chloride (32 equivalents per 10<sup>4</sup> gm.) without formaldehyde showed a molecular weight of 38,000.

|| Preparations partly insoluble.



The data show that ammonia and bivalent primary and secondary amines can serve as cross-linking agents under these conditions (Table III). The remarkable effect of traces of such amines in causing the rapid gelation of zein- (16) and gliadin- (5) formaldehyde mixtures has been previously discussed and explained on the basis of reaction I (5). The present data appear to supply the proof for the formation of intermolecular cross-links under similar conditions. Here again, the insolubility of the preparations presumably containing the most cross-links defeats, to a certain extent,

the purpose of demonstrating the extent of polymerization in a quantitative manner by osmometry. Furthermore, indications were obtained that certain cross-links of the type resulting from reaction I (*e.g.*,  $R = CH_3-CH-COOH$ ) are rather unstable and may be broken in the course of prolonged dialysis. These facts may explain why increased molecular weights were found only if the reaction had been performed under specific conditions of concentration and time and with certain catalytic amines, whereas

TABLE IV

*Apparent Molecular Weights of Soluble Preparations of Gliadin Treated with Formaldehyde in Presence or Absence of Various Amines in 45 Per Cent Ethanol\**

Added compound†	Final pH	Apparent mol. wt.‡
None.....	5.6-7.8	38,000
Ammonium chloride.....	5.4	46,000
“ acetate.....	5.2	47,000
Methylamine, HCl.....	5.6	76,000§
	6.0	63,000
	6.4	30,000§
Dimethylamine, HCl.....	5.6	41,000
Ethanolamine.....	5.8	37,000§
Diethanolamine.....	5.8	43,000
Glycine.....	5.6	61,000
Alanine.....	5.7	42,000
Proline.....	6.3	35,000

\* The concentrations of protein and formaldehyde were 4 and 7.5 per cent, respectively. The reaction time was 2 days. The desired pH was obtained by addition of small amounts of N sodium hydroxide and hydrochloric acid.

† 32 equivalents per 10<sup>4</sup> gm. of protein.

‡ Osmotic pressure measurements performed as in Table III.

§ Products partly insoluble.

the phenomenon of gelation was found to occur much more generally and with almost all primary amines (5).

Another series of experiments with gliadin was performed in 45 per cent ethanol at pH 5.5 to 6.5. Under such conditions formaldehyde alone yielded a gliadin derivative having a molecular weight of 38,000, but in the presence of methylamine, glycine, or ammonium salts increases in the molecular weights of the reaction products resulted (Table IV). Secondary amines had no such cross-linking effect.

The results obtained with gliadin are paralleled by those obtained with polyglutamine. This polypeptide also contains a great number of primary amide and very few amino groups and, therefore, it is not readily



cross-linked by formaldehyde.<sup>5</sup> Again, the addition of ammonium salts or methylamine to the reaction mixture caused increases in the apparent molecular weight of the polypeptide (Table V). That primary amines may enter into cross-links even in proteins containing amino groups is indicated by the results obtained upon addition of alanine or methylamine to serum albumin-formaldehyde reaction mixtures (Table I, foot-note).

TABLE V

*Apparent Molecular Weights of Soluble Preparations of Polyglutamine Treated with Formaldehyde in Presence or Absence of Various Amines*

Reaction conditions*			Apparent mol. wt.†
Added compound‡	Time	Final pH	
	<i>days</i>		
None.....	5	3.3	20,000
Ammonium chloride (2).....	5		37,500
"    acetate (80).....	5	3.6	34,000
Methylamine, HCl (80).....	1	2.6	27,000
Dimethylamine, HCl (80).....	5	3.2	19,000
Alanine (80).....	5	3.3	19,000
"    HCl (80).....	5	2.8	19,000
Proline.....	5	3.3	20,000

\* The reactions were performed in 1.5 M acetic acid. The concentrations of polyglutamine and formaldehyde were 5 and 4 per cent, respectively.

† The figures in parentheses represent equivalents used per 10<sup>4</sup> gm. of polyglutamine.

‡ Osmotic pressure measurements performed in 0.05 M citrate-0.05 M NaCl-6.7 M urea, pH 5.8 to 6.4.

## SUMMARY

Formaldehyde treatment at pH 3 to 8 and room temperature of concentrated solution of proteins containing amino groups led to marked increases in the molecular weights, as measured by the osmotic pressure of the (water-soluble) reaction products. Amino-acetylated proteins are not similarly affected.

Any tendency to aggregate was minimized by performing the osmotic pressure measurements in 7 to 10 M urea solutions. It was, therefore, concluded that the formation of methylene bridges between the amino and other reactive groups of two or more protein molecules represents the chemical basis for the observed increases in molecular weights.

<sup>5</sup> As in the case of gliadin, some increases in the molecular weight are obtained at high concentrations in 50 per cent acetic acid, in this case even in the absence of formaldehyde. The latter phenomenon may be due to the formation of imide cross-links between two carboxyl groups.

Proteins or polypeptides rich in amide but poor in amino groups were not readily cross-linked under these conditions, but increased molecular weights could be produced by the addition of functionally divalent amino compounds which provide bridges between pairs of amide groups.

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## LETTERS TO THE EDITORS

### ON THE MODE OF ACTION OF BIOTIN

Sirs:

Under this title, Axelrod *et al.*<sup>1</sup> have recently published a note confirming the observations of Lichstein and Umbreit<sup>2</sup> that incubation of freshly harvested cell suspensions of *E. coli* in molar phosphate, pH 4, 37°, resulted in

#### *Influence of Growth Medium on Biotin Activation of Phosphate-Treated Bacterial Cells*

Organism	Additions, 0.1 γ per tube	Peptidase*	Tryptone†
		Ammonia N produced	
<i>E. coli</i> (Gratia)	None	γ 3.0	γ 0.2
	Biotin	5.7	0.2
	None	3.8	3.6
	Biotin	5.3	3.6
	None	5.5	2.7
	Biotin	7.9	2.5
	None	3.4	3.0
	Biotin	6.8	3.6
<i>B. cadaveris</i>	None		3.8
	Biotin		6.8
	None		1.8
	Biotin		4.6

\* 1 per cent each of peptidase and yeast extract, and 0.5 per cent  $K_2HPO_4$ .

† The same as above, with peptidase replaced by tryptone. Cells grown in respective media at 30° for 16 hours. Harvested cells aged in M phosphate, pH 4, 30 to 45 minutes at 22–25°. Reaction run at pH 4, 37°, 30 to 45 minutes; volume, 2 ml. DL-Aspartic acid added at 0.005 M final concentration. The figures represent micrograms of ammonia nitrogen produced after subtracting the amount present in similar cell suspensions incubated without added aspartate.

marked loss of activity with respect to ammonia and carbon dioxide production from added aspartic acid. They were, however, unable to reactivate such cells by the addition of biotin, although hot water extracts from normal, and even biotin-deficient, cells were able to reactivate them. They

<sup>1</sup> Axelrod, A. E., Hofmann, K., Purvis, S. E., and Mayhall, M., *J. Biol. Chem.*, **175**, 991 (1948).

<sup>2</sup> Lichstein, H. C., and Umbreit, W. W., *J. Biol. Chem.*, **170**, 329, 423 (1947).

therefore concluded that it was very unlikely that biotin plays any rôle in these enzyme systems.

Consideration of the data presented and discussions with Dr. Axelrod revealed that the primary difference in the experiments was in the medium employed to grow the organism. Lichstein and Umbreit<sup>2</sup> employed peptidase; Axelrod *et al.*<sup>1</sup> used tryptone or peptone. Through the courtesy of Dr. C. R. Brewer we obtained a sample of peptidase similar to that previously employed. When grown on a medium containing peptidase the Gratia strain of *E. coli* (employed in both groups of studies) responds to the addition of biotin after inactivation, while, when grown on a tryptone medium, biotin alone is ineffective (see the table).

It is further apparent, however, that other strains of bacteria (as *B. cadaveris*, *P. vulgaris*) show a biotin response when grown on tryptone medium; hence the requirement for peptidase appears to apply largely to the Gratia strain of *E. coli*.

While the reactions resulting in inactivation by exposure to molar phosphate at pH 4 are not yet understood, and are not always consistent, since doubtless they are influenced by a variety of factors, it would seem unsafe in view of the positive data already presented<sup>2, 3</sup> to conclude that biotin is not involved in these reactions.

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Received for publication, November 10, 1948

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<sup>3</sup> Lichstein, H. C., and Christman, J. F., *J. Biol. Chem.*, 175, 649 (1948).

## HEMIN SYNTHESIS IN RABBIT BONE MARROW HOMOGENATES\*

Sirs:

It has been shown by Altman *et al.*<sup>1</sup> that glycine labeled with C<sup>14</sup> in the  $\alpha$ -carbon atom is a precursor of hemin. Evidence of *in vitro* synthesis of hemin has been reported by Shemin *et al.*,<sup>2</sup> who showed that the glycine labeled with N<sup>15</sup> is incorporated into hemin by circulating avian erythrocytes. The data to be presented here demonstrate that hemin is synthesized by rabbit bone marrow homogenates.

Time of incubation <i>hrs.</i>	Protoporphyrin dimethyl ester, 10 <sup>4</sup> disintegrations per minute per mm	C <sub>0</sub> :C*
0.5	0.00	
1.5	1.52	36.0
3	12.55	13.7
17	40.05	3.1
44	54.50†	9.8

\* Ratio of initial isotope concentration of glycine (C<sub>0</sub>) to isotope concentration of protoporphyrin dimethyl ester isolated (C).

† Isolated as hemin.

Bone marrow homogenates were prepared and incubated as previously described.<sup>3</sup> Bone marrow obtained from three rabbits was used for each experiment. After termination of the incubation period, the homogenate was centrifuged at high speed in order to separate the fats from the remainder of the preparation. The latter, a reddish colored suspension, was treated with 3 volumes of acetone. The acetone precipitate thus obtained was dried and protoporphyrin dimethyl ester was isolated from it by the method of Grinstein.<sup>4</sup> The results obtained are presented in the table.

The results indicate that rabbit bone marrow homogenates can synthesize hemin from methylene-labeled glycine. By using C<sup>14</sup>-labeled glycine,

\* This paper is based on work performed under contract with the United States Atomic Energy Commission at The University of Rochester, Atomic Energy Project, Rochester, New York.

<sup>1</sup> Altman, K. I., Casarett, G. W., Masters, R. E., Noonan, T. R., and Salomon, K., *J. Biol. Chem.*, **176**, 319 (1948).

<sup>2</sup> Shemin, D., London, I. M., and Rittenberg, D., *J. Biol. Chem.*, **173**, 799 (1948).

<sup>3</sup> Altman, K. I., in press.

<sup>4</sup> Grinstein, M., *J. Biol. Chem.*, **167**, 515 (1947).

unequivocal evidence of appreciable protein synthesis in these bone marrow homogenates has been obtained,<sup>5</sup> although it cannot be stated with certainty at this time whether or not globin synthesis occurs.

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Received for publication, November 11, 1948

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<sup>5</sup> Unpublished experiments.

# GENETIC DIFFERENCES IN METHIONINE UPTAKE BY SURVIVING TISSUE\*

Sirs:

Recent research in physiological genetics has established the genetic control of certain major biochemical processes in lower organisms. The results we wish to report here show the operation of a similar genetic control of the protein metabolism of a higher animal.

500 mg. of liver slices; 5 ml. volume; incubation 2 hours at 36°; 95 per cent O<sub>2</sub>-5 per cent CO<sub>2</sub>. Each test represents six or more samples.

Date tested	J strain				F strain			
	Pooled tissue from	Average weight	S. A.*	Standard uptake†	Tissue from	Average weight	S. A.*	Standard uptake†
		gm.		per cent		gm.		per cent
May 12	2 ♂	54	50	1.90				
October 1	3 ♂	60	46	1.65	3 ♂	62	121	4.10
April 10	4 ♂	105	24	0.85	4 ♂	87	102	3.45
					3 ♂	94	91	3.10
	5 ♀	91	40	1.40	4 ♀	75	115	3.95
					4 ♀	82	107	3.60
September 13	2 ♂	150	55	1.85	2 ♂	220	93	3.30
May 12	2 ♂	215	30	1.10	2 ♂	230	93	3.15

\* Counts per minute

$\mu\text{M}$  methionine

†  $\frac{\text{Counts per minute uptake}}{\text{Counts per minute used}} \times \frac{\text{assumed } 17 \mu\text{M methionine in sample}}{\text{actual methionine recovered}}$  (calculated from the data of Lee, W. C., and Lewis, H. B., *J. Biol. Chem.*, 107, 649 (1934)).

Two inbred strains of rats were compared as to their ability to incorporate methionine into surviving liver slices. The strains differed significantly in body weight and their reciprocal cross offspring showed marked heterosis. The J strain was built up from four females and two males of a single litter of the Wayne University Fisher line No. 344, with a pedigree of forty-five generations of sister and brother mating. The F strain was started from two pregnant females of the highly inbred King A inbred albino strain of the Wistar Institute and has been continued by sister and brother mating.

\* Aided by grants from the American Cancer Society (recommended by the Committee on Growth of the National Research Council) and carried on during the tenure, by R. R., of a research assistantship in the Division of Genetics, College of Agriculture, University of California.

Separate pools of liver slices from male or female animals were prepared substantially according to Melchior and Tarver.<sup>1</sup> The pooled slices were incubated in Ringer's-bicarbonate containing  $1.33 \mu\text{M}$  of methionine labeled with  $\text{S}^{35}$  at the level of  $5 \times 10^4$  counts per minute. After incubation, the tissues were heat-denatured, washed, and the methionine content and radioactivity determined according to the method of Simpson and Tarver.<sup>2</sup>

The results (summarized in the table) show a consistent and surprisingly large difference between the two strains in the incorporation of methionine into liver protein. This large difference was obtained in tests carried on at different times and involving animals of different generations. The standard uptake shows some tendency to decrease with age. The uptake ratio for the two strains, however, did not vary significantly with age.

Nitrogen and sulfur analyses of the livers used and liver to body weight ratios of animals used showed no significant differences. As a consequence, application of the *in vitro* rates to the whole animal indicates that significantly different amounts of liver protein may be synthesized by the two strains under similar dietary conditions.

Complete data on the inbred strains and their reciprocal crosses and back-crosses will be published elsewhere. The  $\text{F}^1$  and the back-cross data cannot be explained solely in terms of gene segregation and appear to require the assumption of a maternal effect.

The results presented here reemphasize the need of guarding against genetic variation in stock laboratory animals and of designing biochemical experiments so that genetic variations are detected.

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Received for publication, November 29, 1948

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<sup>1</sup> Melchior, J. B., and Tarver, H., *Arch. Biochem.*, **12**, 301 (1947).

<sup>2</sup> Simpson, M., and Tarver, H., unpublished data.



# COENZYME A AS GROWTH STIMULANT FOR ACETOBACTER SUBOXYDANS\*

Sirs:

It had been found that pantothenic acid bound in coenzyme A and its partial degradation products is inactive in the test with *Lactobacillus arabinosus*.<sup>1</sup> In this connection a recent report by King, Locher, and Cheldelin<sup>2</sup> was of considerable interest. They isolated from heart muscle a pantothenic acid-containing factor which, with *A. suboxydans*, gave a greater and faster growth response than would have been expected from its pantothenic acid content. *L. arabinosus* did not respond to their preparation, except after decomposition with intestinal phosphatase. The similarity of this factor to coenzyme A was pointed out. In contrast it was found, however, that the preparation did not restore sulfanilamide acetylation and appeared difficultly dialyzable.

*A. suboxydans* ATCC 621 was grown in the pantothenic acid-free medium of Sarett and Cheldelin.\* The density readings recorded below were taken after 40 hours on the Klett colorimeter. The coenzyme A preparation used contained 100 units per mg., corresponding to a pantothenic acid content of 6.5 per cent.

Compound	Enzyme treatment	Pantothenic acid equivalent added	
		0.7 $\gamma$	1.4 $\gamma$
Free pantothenic acid	0	4	12
Coenzyme A	0	70	130
“ “	Liver extract	55	125
“ “	Intestinal phosphatase	6	18

\* Sarett, H. P., and Cheldelin, V. H., *J. Biol. Chem.*, **159**, 311 (1945).

It is shown in the present report that purified preparations of coenzyme A and the product of its degradation with liver extract exhibit a stimulatory effect on *A. suboxydans* in excess of their pantothenic equivalent (*cf.* the table). This effect disappears upon treatment with intestinal phosphatase. As with the factor of Cheldelin and his group, the growth response is faster with coenzyme A than with free pantothenic acid. Proportionality between the enzymatic and the growth effects of coenzyme A was found with

\* Aided by a grant from the Commonwealth Fund.

<sup>1</sup> Lipmann, F., Kaplan, B. O., Novelli, G. D., Tuttle, L. C., and Guirard, B. M., *J. Biol. Chem.*, **167**, 869 (1947).

<sup>2</sup> King, T. E., Locher, L. M., and Cheldelin, V. H., *Arch. Biochem.*, **17**, 483 (1948); Abstracts, American Chemical Society, 114th meeting, 59C (1948).

preparations of various degrees of purity ranging from 30 to 130 units per mg.<sup>3</sup>

The similarities of intact and partially degraded coenzyme A and of the activator of Cheldelin and his group induced us to recheck the rate of dialysis of coenzyme A through cellophane, which we found surprisingly variable. During 18 hours of dialysis through cellophane at 5° a crude rabbit liver extract lost 66 per cent activity, while a purified preparation with an activity of 100 units<sup>3</sup> per mg. lost only 33 per cent. The latter preparation diffused about 1½ times faster through collodion. This variability most likely should be due to impurities affecting pore size and charge. In unpublished experiments the molecular weight of coenzyme A has been determined with the sintered glass diffusion cell of Northrop and values were obtained of  $800 \pm 50$ . The results reported here indicate that the stimulation of *A. suboxydans* discovered by Cheldelin's group should be due to coenzyme A or its degradation product. Partial autolysis in animal organs appears to yield considerable amounts of this still phosphorylated, but enzymatically inactive, intermediary.

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Received for publication, November 30, 1948

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\* Kaplan, N. O., and Lipmann, F., *J. Biol. Chem.*, **174**, 37 (1948).

## OROTIC ACID, A PRECURSOR OF PYRIMIDINES IN THE RAT *Sirs:*

In recent years orotic acid (uracil-4-carboxylic acid) has been found to be essential for the growth of some microorganisms;<sup>1</sup> some pyrimidine-requiring *Neurospora* mutants were found to grow equally well with uracil and orotic acid<sup>2</sup> and some mutants have been found to produce orotic acid.<sup>3</sup>

It has been suggested that orotic acid is a by-product in the biosynthesis of pyrimidines in *Neurospora*.<sup>3</sup>

In order to investigate the metabolism of orotic acid in the rat we have synthesized orotic acid<sup>4</sup> containing 6.06 atom per cent excess N<sup>15</sup>. This acid was injected into rats, 12.5 mg. per 100 gm. of body weight, twice daily for 3 days.

From the pooled livers the polynucleotides were prepared and the ribosides isolated.

The cytidine and uridine were found to contain respectively 0.872 and 1.133 atom per cent excess N<sup>15</sup>; *i.e.*, a dilution of only 5.4- and 6.9-fold.

The purine ribosides, adenine and guanine from DNA and the proteins contained only insignificant amounts of N<sup>15</sup>.

Plentl and Schoenheimer<sup>5</sup> have found that neither uracil nor thymine administered to rats was utilized for the synthesis of polynucleotides.

Our results prove that the injected orotic acid was utilized in the biosynthesis of both uracil and cytosine.

A detailed report will appear later.

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Received for publication, December 2, 1948

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<sup>1</sup> Rogers, H. J., *Nature*, **153**, 251 (1944).

<sup>2</sup> Loring, H. S., and Pierce, J. G., *J. Biol. Chem.*, **153**, 61 (1944).

<sup>3</sup> Mitchell, H. K., Houlahan, M. B., and Nyc, J. F., *J. Biol. Chem.*, **172**, 525 (1948).

<sup>4</sup> Nyc, J. F., and Mitchell, H. K., *J. Am. Chem. Soc.*, **69**, 1382 (1947).

<sup>5</sup> Plentl, A. A., and Schoenheimer, R., *J. Biol. Chem.*, **153**, 203 (1944).



COMPLETE FRACTIONATION OF BRAIN CEPHALIN:  
ISOLATION FROM IT OF PHOSPHATIDYL SERINE,  
PHOSPHATIDYL ETHANOLAMINE, AND  
DIPHOSPHOINOSITIDE

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(Received for publication, July 31, 1948)

Brain cephalin (1) has been the name given to a fraction of brain phosphatides characterized by its solubility in ether and insolubility in ethyl alcohol. For many years it was assumed to be a single compound with the chemical structure of diacylglycerolphosphoryl ethanolamine. Work from this laboratory has shown brain cephalin to be a mixture (2). From it were separated two pure phosphatides; namely, phosphatidyl ethanolamine and phosphatidyl serine. The former phosphatide has the chemical structure classically attributed to brain cephalin and the latter has been found to be oleylstearyl glycerylphosphoryl serine (3). Besides these two phosphatides, a material was obtained which appeared to be a mixture and to which the name of inositol phosphatide was given for descriptive purposes, since it had inositol as a constituent. This paper contains detailed proof that inositol phosphatide is a mixture and describes a method for the separation from it of phosphatidyl ethanolamine, phosphatidyl serine, and a new inositol-containing phosphatide to which the name of diphosphoinositide has been given. Results establishing in part the chemical structure of diphosphoinositide have already been reported in a preliminary note (4) and are given in detail in the accompanying paper (5).

The evidence here reported establishes that brain cephalin is a mixture of the three phosphatides mentioned above. It contains only small amounts of other substances which can be considered as contaminants. These contaminants are either lipides of the carbohydrate-containing type or non-lipide water-soluble substances among which phosphates appear to be predominant. The water-soluble contaminants can be removed by dialysis.

Brain cephalin, as defined by its solubilities, does not account for all of the lipide  $\text{NH}_2\text{-N}^1$  present in brain. Not an inconsiderable amount of

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<sup>1</sup> The term  $\text{NH}_2\text{-N}$  is used to indicate aliphatic amino nitrogen determinable by the nitrous acid method of Van Slyke (6).

lipide  $\text{NH}_2\text{-N}$  can be found in the supernatant solutions obtained in the course of preparation of brain cephalin. This lipide  $\text{NH}_2\text{-N}$  appears to be phosphatidyl ethanolamine, which is to be expected since this phosphatide is soluble in alcohol (2). On the other hand, brain cephalin appears to account for most of the carboxyl  $\text{N}^2$  present in brain. Only small amounts of lipide carboxyl  $\text{N}$  are found in brain lipide fractions other than brain cephalin.

The method of fractionation of the material previously called inositol phosphatide into its different components is the result of many unfruitful attempts. In the earlier part of the work we were handicapped by our lack of knowledge of the chemical nature of the components of the mixture. This prevented the setting up of reliable criteria for the value of the different methods of fractionation that were attempted. Inositol content of the fractions, which was the only way of estimating progress towards isolation of the pure inositol-containing phosphatide, can only be determined by bioassay. This bioassay could only show fairly large differences in inositol content of different fractions obtained. As will become apparent to the reader, the method of fractionation finally developed yields fractions of different inositol content, but the differences at each step are so small that inositol determinations by bioassay would fail to show it. A more reliable criterion was finally provided by the isolation of inositol metaphosphate from inositol phosphatide (described in the accompanying paper (5)), since this finding allowed the assumption that the pure inositol-containing phosphatide would show a phosphorus content larger than that found in known phosphatides. With this idea in mind, fractions obtained from inositol phosphatide by the different methods of fractionation tried were analyzed for phosphorus. The use of the phosphorus content as a criterion led to the fractionation method described below.

This method is based on the fact that diphosphoinositide is less soluble in methyl alcohol than either phosphatidyl serine or phosphatidyl ethanolamine. This difference in solubility is less evident when mixtures are being dealt with. Therefore, it is necessary to repeat the treatment with methyl alcohol a number of times before essentially pure diphosphoinositide is obtained. From the filtrates it is easy to separate phosphatidyl serine and phosphatidyl ethanolamine by means of their different solubility in ethyl alcohol (2).

Phosphatidyl serine and phosphatidyl ethanolamine have been identified by isolating among their cleavage products L-serine from the former and ethanolamine from the latter. Glycerophosphoric acid had been isolated

<sup>2</sup> The term  $\text{COOH-N}$  or carboxyl nitrogen is used to indicate aliphatic amino acid nitrogen determinable by the ninhydrin method of Van Slyke, Dillon, MacFadyen, and Hamilton (7).

previously from inositol phosphatide (2). Therefore, the two compounds appear to be identical with, or closely related to, those isolated from brain cephalin by a former method (3). Phosphatidyl serine isolated from inositol phosphatide is mainly in the form of a sodium salt, in contrast with that obtained earlier (3) which is mainly in the form of a potassium salt.

#### EXPERIMENTAL

*Analytical Methods*—The methods used are described elsewhere (2, 3).

*Preparation of Inositol Phosphatide*—Cephalin is prepared from beef brain by the method described earlier (2). It is then fractionated by the chloroform-alcohol method (2), modified as described below.

1 gm. of cephalin is dissolved in 8 cc. of  $\text{CHCl}_3$  and to the clear solution are added 11.8 cc. of ethyl alcohol (1.45 as much alcohol as chloroform, by volume). A turbidity develops, and on standing, or by centrifugation, the mixture resolves itself into a viscous underlayer (inositol phosphatide fraction) and a clear supernatant solution. The supernatant solution (which contains most of the phosphatidyl serine and phosphatidyl ethanolamine present in the original cephalin) is decanted and the viscous underlayer is treated with ethyl alcohol. A solid precipitate that forms is collected on a Büchner funnel and dried.

The yield of inositol phosphatide is about 5 gm. per kilo of initial tissue. The preparation corresponds to the combination of Fractions I and II as obtained by the chloroform-ethanol method first described (2). The composition of these fractions has already been given (Table I, Columns 2 and 3 (2)). The preparation contains all the inositol present in the starting cephalin. It is, however, a complex mixture which on analysis is found to contain C 53.0 per cent, P 5.38 per cent, inorganic P 1.02 per cent, total N 1.36 per cent,  $\text{NH}_2\text{-N}$  1.33 per cent,  $\text{COOH-N}$  0.87 per cent, carbohydrate (as galactose) from 0.7 to 1.3 per cent, Na 2.04 per cent, K 3.60 per cent, Ca 0.15 per cent, Mg 0.45 per cent.

#### *Fractionation of Inositol Phosphatide by Chloroform-Methanol Method*

The method to separate from inositol phosphatide its different components is as follows:

The whole procedure is run at about 4°. 1 gm. of inositol phosphatide is dissolved in 12 cc. of chloroform and to the clear solution are added 22 cc. of methanol. A precipitate forms immediately. The mixture is shaken in a shaking machine for 30 minutes, after which the precipitate separates easily, leaving a water-clear supernatant solution. The precipitate is collected on a Büchner funnel or by centrifugation and is transferred back to the container in which the precipitation was carried out. The precipitate is redissolved in 12 cc. of chloroform (part of the chloroform can be

used to return the precipitate to the original container), 22 cc. of methanol are added to the clear solution, and the procedure repeated exactly as before. The whole operation is repeated for as long as the supernatant solution contains material which on analysis is found to contain <4.5 per cent P. The supernatant solution must be clear, as any cloudiness is due to suspended diphosphoinositide. Material recovered from cloudy supernatant solutions is found to contain on analysis from 4.5 to 7 per cent P. Occasionally it happens that the chloroform-methanol-insoluble precipitate does not go completely into solution in chloroform. The chloroform-insoluble material can neither be removed by centrifugation nor by filtration. It can be eliminated by adding 2 volumes of ethyl ether to the turbid chloroform solution and collecting by centrifugation whatever insoluble material there is. In one case, in which it was studied, the insoluble material represented a very small fraction of the total preparation and had the composition of phosphatidyl serine. The residue obtained on evaporation in a vacuum of the clear chloroform-ethyl ether solution was completely soluble in chloroform. The chloroform-methanol treatment was then resumed.

It has been found that the procedure runs more smoothly and requires a smaller number of successive precipitations when the original inositol phosphatide has not been freed of water-soluble impurities by dialysis. With the non-dialyzed inositol phosphatide, twelve successive precipitations usually suffice, whereas, with the inositol phosphatide that has been purified by dialysis, as many as thirty successive precipitations are required.

The different supernatant solutions are labeled IP<sub>1</sub>, IP<sub>2</sub>, and so on, in the order in which they are obtained. In all cases the solvents are removed by evaporation in a vacuum. Therefore, from the original inositol phosphatide there are obtained a number of IP fractions and a final precipitate which contains all of the diphosphoinositide grossly contaminated, especially with inorganic phosphates. The yield of impure diphosphoinositide is about 20 per cent of the original inositol phosphatide, or 1 gm. per kilo of starting brain tissue.

Data on yields for different fractions and results of their chemical analyses are given in Table I.

### *Study of IP Fractions*

From data given in Table I it appears that all IP fractions contain about 3.5 per cent P and 1.4 per cent N, the higher values for P shown by fractions IP<sub>9</sub> to IP<sub>12</sub> being due to contamination by inorganic phosphates. Practically all of the nitrogen is NH<sub>2</sub>-N. Otherwise, the fractions appear to fall into three groups: (1) fractions IP<sub>1</sub> and IP<sub>2</sub> that contain only part of



their  $\text{NH}_2\text{-N}$  as carboxyl N; (2) fractions from  $\text{IP}_3$  to  $\text{IP}_8$  that have all of their  $\text{NH}_2\text{-N}$  as carboxyl N; and (3) fractions from  $\text{IP}_9$  on that appear to be the same as fractions  $\text{IP}_3$  to  $\text{IP}_8$  contaminated with inorganic phosphates and, possibly, diphosphoinositide. The study of these fractions detailed below establishes that fractions  $\text{IP}_1$  and  $\text{IP}_2$  are mixtures of phosphatidyl serine and phosphatidyl ethanolamine, fractions  $\text{IP}_3$  to  $\text{IP}_8$  are phosphatidyl serine, and fractions from  $\text{IP}_9$  on are also phosphatidyl serine.

TABLE I

*Fractionation of Inositol Phosphatide by Chloroform-Methanol Method. Composition of Starting Inositol Phosphatide, of Successive Chloroform-Methanol-Soluble Fractions (Supernatants  $\text{IP}_x$ ), and of Diphosphoinositide Finally Obtained Therefrom*

Material*	Yield, per cent of starting material of inositol phosphatide	Components				
		P	Inorganic P	N†	COOH-N	$\frac{\text{COOH-N}}{\text{N}}$
		per cent	per cent	per cent	per cent	
Inositol phosphatide....		5.38	1.02	1.36	0.87	0.632
$\text{IP}_1$ .....	28.4	3.60	0.00	1.39	0.95	0.683
$\text{IP}_2$ .....	17.6	3.40	0.00	1.38	1.322	0.958
$\text{IP}_3$ .....	7.9	3.64	0.00	1.45	1.443	0.995
$\text{IP}_9$ .....	2.00	4.62	0.30	1.27	1.22	0.960
$\text{IP}_{12}$ .....	0.70	5.56	1.20		0.97	
Diphosphoinositide.....	19.0	11.06	6.20	0.78	0.20	0.251

\* Fractions  $\text{IP}_4$  to  $\text{IP}_8$  had the same composition as  $\text{IP}_3$ . The yields were, respectively, 6.1, 4.8, 3.6, 3.9, and 2.7 per cent of starting inositol phosphatide. Fractions  $\text{IP}_{10}$  and  $\text{IP}_{11}$  had compositions ranging between those of  $\text{IP}_3$  and  $\text{IP}_{12}$ . The yields were, respectively, 1.1 and 0.8 per cent of starting inositol phosphatide.

† All fractions from  $\text{IP}_1$  to  $\text{IP}_{12}$  had all of their N as  $\text{NH}_2\text{-N}$ .

*Fractionation of  $\text{IP}_1$  and  $\text{IP}_2$  Fractions and Identification of Phosphatides Separated Therefrom*—From these fractions, either singly or combined, two fairly pure phosphatides can be obtained, one which contains all of its N as  $\text{NH}_2\text{-N}$  in non-amino acid form (low carboxyl N phosphatide), and a second which contains all of its N as carboxyl N (high carboxyl N phosphatide). The method of separation is based on the fact that the former phosphatide is much more soluble in ethyl alcohol than the latter (2).

17.6 gm. of combined  $\text{IP}_1$  and  $\text{IP}_2$  were dissolved in 160 cc. of chloroform and 600 cc. of ethyl alcohol were added to the clear solution. On standing overnight in the ice box, a precipitate separated which was collected and dried. It weighed 12.6 gm. (71.6 per cent of starting material) and on analysis was found to contain P 3.5 per cent, N 1.35 per cent, carboxyl N 1.32 per cent (high carboxyl N phosphatide).

Material left in the supernatant solution was recovered by removal of the solvent by vacuum distillation. After drying to constant weight, the material obtained weighed 5 gm. (28.4 per cent of starting material) and on analysis was found to contain P 3.5 per cent, N 1.4 per cent,  $\text{NH}_2\text{-N}$  1.4 per cent, carboxyl N 0.15 per cent (low carboxyl N phosphatide).

*Isolation of Ethanolamine from Low Carboxyl N Phosphatide*—2.0 gm. of low carboxyl N material obtained as described above were emulsified in 150 cc. of  $\text{H}_2\text{O}$ . Concentrated HCl was added to the chilled emulsion until the precipitation of the phosphatide appeared to be complete. 70 cc. of concentrated HCl were required. This is in sharp contrast to the behavior of the high carboxyl N phosphatide which precipitates quantitatively out of emulsion with a concentration of HCl below 0.1 N. The precipitate was removed by centrifugation, washed once with 220 cc. of 4 N HCl, and next hydrolyzed by boiling 4 N HCl under a reflux for 2 hours. After cooling, the hydrolysate was filtered and the filtrate was evaporated to dryness in a vacuum. The dry residue was taken up in 35 cc. of water, and the solution was treated with an excess of 25 per cent aqueous solution of neutral lead acetate. The insoluble lead salts were removed by filtration and the filtrate treated with  $\text{H}_2\text{S}$ . The lead sulfide that formed was removed by filtration and the filtrate was evaporated to dryness in a vacuum. The residue weighed 250 mg. and contained 18.6 mg. of  $\text{NH}_2\text{-N}$ .

The picrolonate was prepared by dissolving the theoretical amount of picrolonic acid required (350 mg.) and the residue in 30 cc. of boiling water (8). About one-tenth of the picrolonate formed proved insoluble, despite prolonged stirring. The insoluble material was allowed to settle and the clear supernatant was decanted into another tube and let cool slowly. After standing overnight in the ice box, the crystals that had formed were collected. After drying, the crystals weighed 350 mg. and on analysis were found to contain 3.27 per cent  $\text{NH}_2\text{-N}$  and 0.06 per cent carboxyl N. The crystals were recrystallized twice from boiling water without significant change in the  $\text{NH}_2\text{-N}$  content. They were then washed with ether. After drying, they were found to contain 3.95 per cent  $\text{NH}_2\text{-N}$ ; theory for ethanolamine picrolonate, 4.31 per cent.

This material was combined with that recovered from the supernatant solutions of the two recrystallizations. The combined material was treated with hot dilute HCl, which resulted in precipitation of most of the picrolonic acid. The filtrate from the picrolonic acid precipitate was freed of residual picrolonic acid by shaking with ether. The water-clear dilute hydrochloric acid solution was evaporated to dryness in a vacuum and a crystalline residue was obtained which weighed 70 mg. On analysis it was found to contain  $\text{NH}_2\text{-N}$  14.25 per cent, carboxyl N 0.00 per cent, and C 24.6 per cent. Theory for ethanolamine hydrochloride is  $\text{NH}_2\text{-N}$  14.36

per cent and C 24.6 per cent. Allowing for the aliquots used for analysis, the amount of ethanalamine hydrochloride isolated accounted for over 50 per cent of non-amino acid  $\text{NH}_2\text{-N}$  in the starting phosphatide.

*Isolation of L-Serine from High COOH-N Phosphatide*—3.7 gm. of this phosphatide were freed of bases by emulsification in 200 cc. of water and precipitation of the phosphatide by addition of HCl up to 0.1 N concentration. The precipitate was washed once with 0.1 N HCl and next hydrolyzed by boiling 6 N HCl under a reflux for 3 hours. After cooling, the hydrolysate was filtered and the filtrate dried by evaporation in a vacuum. The residue was dissolved and the solution was decolorized with charcoal. The filtrate from the charcoal was found to contain 35.4 mg. of carboxyl N. The solution was concentrated to a volume of 11 cc. and to it were added 660 mg. of *p*-hydroxyazobenzene-*p*-sulfonic acid (9), which was dissolved by heating in the boiling water bath. Crystals that formed on standing overnight in the ice box were collected. After drying, they weighed 700 mg. The crystals were dissolved in water, the *p*-hydroxyazobenzene-*p*-sulfonic acid was precipitated by addition of excess neutral lead acetate, and the insoluble lead salt removed by filtration. The filtrate was treated with charcoal to remove the last traces of *p*-hydroxyazobenzene-*p*-sulfonic acid and the filtrate from the charcoal was treated with  $\text{H}_2\text{S}$ . The filtrate from the lead sulfide was concentrated to a volume of 3 cc. and treated with 40 cc. of ethyl alcohol. On standing overnight, sharp needles separated.

The crystals were collected and, after drying at  $100^\circ$ , weighed 140 mg. On analysis they proved to be L-serine. They accounted for over 60 per cent of the carboxyl N in the starting phosphatide. Results of analyses were as follows:

$\text{C}_3\text{H}_7\text{O}_3\text{N}$ . Calculated.	C 34.2, H 6.67, N 13.32, carboxyl N 13.32
Found (corrected for 0.4% ash).	" 34.2, " 6.62, " 13.27, " " 13.21

*Rotation*—A solution in 1 N HCl containing 50 mg. of crystals per cc. showed in a 1 dm. tube a rotation of  $+0.70^\circ$  with sodium light;  $[\alpha]_D^{20} = +14.2^\circ$ . Fischer and Jacobs give  $+14.5^\circ$  (10).

*Isolation of L-Serine from Combined  $\text{IP}_3$  to  $\text{IP}_8$  Fractions*—By the method described for the high carboxyl N phosphatide, 124 mg. of analytically pure L-serine were obtained from 3 gm. of combined  $\text{IP}_3$  to  $\text{IP}_8$  fractions. This amount accounted for 43 per cent of carboxyl N present in the original material.

*Isolation of L-Serine from Combined  $\text{IP}_9$  to  $\text{IP}_{12}$  Fractions*—From 2.5 gm. of this material, 90 mg. of analytically pure L-serine were obtained by the method described above. This amount of pure L-serine accounted for 48 per cent of carboxyl N present in the original material.

*Bases Combined in Phosphatidyl Serine Separated from Inositol Phosphatide*

Fractions IP<sub>2</sub>, IP<sub>5</sub>, IP<sub>6</sub>, and IP<sub>8</sub> were analyzed for Na, K, Ca, and Mg. They were found to contain an average of 1.7 per cent Na and 1.6 per cent K and to be free of Ca or Mg. The ratio, equivalents of total base to atoms of P, was in all cases about 1.00. In comparison, phosphatidyl serine separated from the original cephalin (3) contains about 3 per cent K and between 0.5 and 1.00 per cent Na.

## SUMMARY

Brain cephalin has been found to be a mixture of phosphatidyl serine, phosphatidyl ethanolamine, and a new inositol containing phosphatide which is called diphosphoinositide.

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# BRAIN DIPHOSPHOINOSITIDE, A NEW PHOSPHATIDE HAVING INOSITOL METADIPHOSPHATE AS A CONSTITUENT

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(Received for publication, July 31, 1948)

Work from this laboratory has established that brain cephalin (1, 2) is a mixture of three different phosphatides; namely, phosphatidylserine (3), phosphatidyl ethanolamine, and diphosphoinositide. In a preliminary note (4) it has been shown that diphosphoinositide has inositol metadiphosphate<sup>1</sup> as a constituent. The present paper contains detailed proof of this statement and reports some other observations which establish in part the chemical structure of diphosphoinositide. The name diphosphoinositide has been chosen in order to emphasize the presence of inositol metadiphosphate in the diphosphoinositide molecule, since this feature sharply differentiates this compound from other known inositol-containing phosphatides, in which inositol and P are present in the ratio of a monophosphate (5-8).

Crude diphosphoinositide is obtained from brain cephalin by a method described in the accompanying paper (2). This crude preparation contains large amounts of water-soluble contaminants, among which inorganic phosphates are predominant. The crude preparation has around 11 per cent phosphorus, half of which proves to be inorganic phosphorus on analysis. It contains large amounts of sodium and potassium and small amounts of calcium and magnesium. By dialysis, it is possible to remove all of the inorganic phosphorus along with all of the sodium and potassium. In one case, in which the dialyzable fraction from the crude diphosphoinositide was analyzed, it was found to contain less than 3 per

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<sup>1</sup> The different carbons in the inositol molecule are designated by the numbers 1 to 6 according to orientation of the valency between C and O atoms; so that each carbon is designated always by the same number. The method by means of which the relative positions of the two phosphoryl radicals has been established in the case of inositol diphosphate from diphosphoinositide shows only that these two radicals are placed in the meta position, without giving any information as to which particular carbons are esterified with the radicals. With this point in mind, the writer uses advisedly the notations ortho, meta, and para (customarily limited to compounds exhibiting a benzene ring), in preference to the notations 1, 2, 1, 3, 1, 4, the use of which could only lead to confusion.

cent carbon and otherwise to give results for a mixture of sodium and potassium phosphates. The ratio, equivalents of base to atoms of P, was about 1.8, which corresponds to the composition of phosphates at the physiological pH.

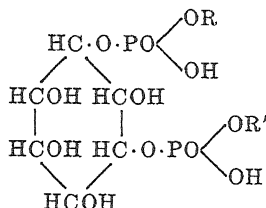
After dialysis, diphosphoinositide is a white gritty powder which is not emulsified in water and is insoluble in most organic solvents with the exception of wet chloroform. Its composition varies slightly from one preparation to another but appears to remain constant for any given preparation on treatment by a large number of solvents or mixtures of solvents that have been tried. It contains on the average 7.3 per cent phosphorus, 0.4 to 0.6 per cent nitrogen, magnesium, and calcium. It also contains small amounts of carbohydrates (between 0.4 and 1.8 per cent in different preparations) and traces of carboxyl N ( $<0.02$  per cent). This suggests the presence of small amounts of contaminants of the cerebroside and phosphatidyl serine types.

The following facts have been established about the chemical structure of diphosphoinositide.

Diphosphoinositide contains as constituents fatty acids, glycerol, and inositol metadiphosphate in approximately equimolar proportions. Inositol metadiphosphate has been isolated from the products of acid hydrolysis of diphosphoinositide and the amount obtained accounts for over 80 per cent of the phosphorus present in the starting material. Therefore, it is reasonable to assume that inositol metadiphosphate is the only type of phosphorus-bearing group present in diphosphoinositide.

Diphosphoinositide is an acidic phosphatide which, on being prepared from brain tissue by the use of neutral solvents, is obtained as a salt of calcium and magnesium with the (equivalents of base)/(atoms of P) ratio of a monophosphate. The K salt of diphosphoinositide can be prepared by treating the Ca-Mg salt with a concentrated solution of potassium oxalate. K diphosphoinositide is a microcrystalline powder. It has the (equivalents of K)/(atoms of P) ratio of a monophosphate. Finally, the bases can be removed from either Ca-Mg diphosphoinositide or K diphosphoinositide by treatment with dilute HCl, which shows that those bases are linked to the diphosphoinositide molecule by a salt-like linkage. The base-free diphosphoinositide is readily soluble in water, giving a transparent solution. The solution is acid, and on titration with standard alkali, uses 1 equivalent of base per atom of P present. All these facts show that the phosphoric acid in diphosphoinositide is present as a diester, with one acid group free for each phosphoryl radical present. Therefore, it can be concluded that the accompanying structure (where R and R<sup>1</sup> stand for unknown radicals) is present in the diphosphoinositide molecule. The total sum of identified radicals (allowing for four ester linkages in the molecule)

accounts for 94 per cent of the starting material. The balance includes bases and contaminants. Therefore, it is believed that all of the molecule is accounted for.

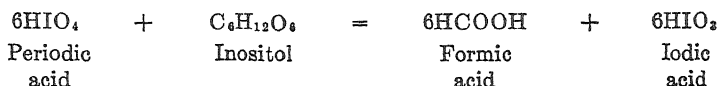


The nature of nitrogen present in diphosphoinositide preparations has not been clarified yet. It is truly lipidic nitrogen, since it cannot be removed by dialysis or by treatment with acid. Part of it can be assigned to cerebrosides, or other carbohydrate-containing lipides. The balance appears to be present as  $\text{NH}_2\text{-N}$ . The analysis for  $\text{NH}_2\text{-N}$  can be run satisfactorily in either the base-free or the potassium salt of diphosphoinositide. Analysis of calcium-magnesium diphosphoinositide fails to give any consistent results, owing presumably to the insolubility of this compound in the reaction mixture. The amount of  $\text{NH}_2\text{-N}$  present has been found to be as low as 0.1 per cent and as high as 0.4 per cent. The same values are obtained before and after acid hydrolysis. The low N:P ratio and the great variation in the amount of  $\text{NH}_2\text{-N}$  present in different preparations suggest that this amine is not an integral part of the diphosphoinositide molecule. On the other hand, the possibility exists that diphosphoinositide is a mixture of two closely related substances, one of which is nitrogen-free, and the second one of which contains an amine as a constituent. The amine present does not appear to be ethanolamine, since several attempts to isolate this substance by different methods have failed.

The chemical structure of inositol metadiphosphate isolated among the products of hydrolysis of diphosphoinositide has been established by (a) elementary analysis, the results of which closely agree with theoretical values calculated from the postulated formula; (b) isolation from it of inositol in theoretical yield; (c) titration to pH 8.2 (phenolphthalein), which shows that each phosphoryl radical in the molecule has two free acid groups; and (d) the study of the products of its reaction with  $\text{HIO}_4$ , which shows that the two phosphoryl radicals on the inositol molecule are in the meta position.

As is well known,  $\text{HIO}_4$  (9) reacts with polyols that exhibit the group  $\text{—CH(OH)—CH(OH)—}$ . The carbon chain is split with oxidation of the alcoholic groups to aldehydes. In the case of compounds exhibiting the group  $\text{—CH(OH)—CH(OH)—CH(OH)—}$  the carbon chain is split on

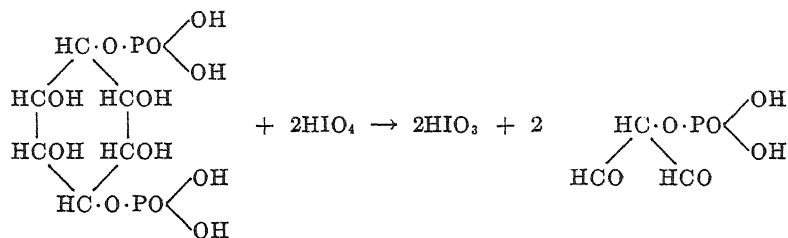
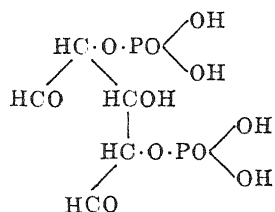
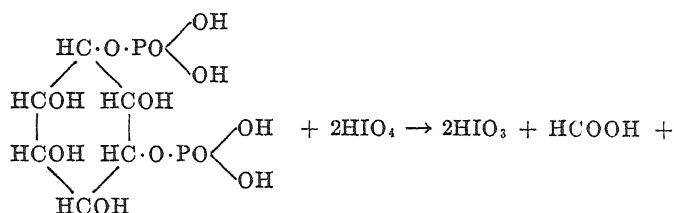
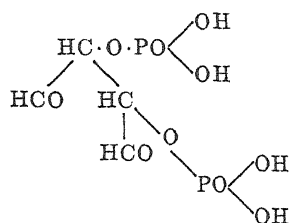
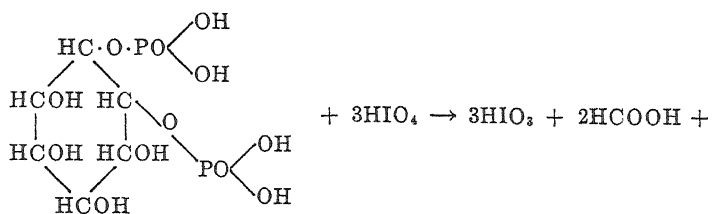
both sides of the middle carbon, the alcoholic group of which is oxidized to  $\text{HCOOH}$ , while the alcoholic groups on the two other carbons are oxidized to aldehydes. With slight excess of  $\text{HIO}_4$ , the reaction is quantitative for all polyols tested (9), 1 mole of  $\text{HIO}_4$  going to  $\text{HIO}_3$  for each bond split. With inositol, Fleury and Joly (10) found that  $\text{HIO}_4$  reacts as follows:



With between 20 and 200 per cent excess  $\text{HIO}_4$  the reaction is complete in 3 hours at room temperature. The amount of  $\text{HCOOH}$  produced amounts to only slightly above 90 per cent of the theoretical yield, supposedly due to side reactions. It is obvious from all this evidence that the amount of  $\text{HIO}_4$  used per mole of inositol diphosphate and the products of the reaction must differ considerably according to the position of the two phosphoryl radicals on the inositol ring, as shown in the accompanying reactions.

With this idea in mind, a method was developed by means of which it was possible to determine in the same solution of inositol diphosphate and  $\text{HIO}_4$  the amount of  $\text{HIO}_4$  used and the amount of  $\text{HCOOH}$  produced. This method is based on the finding of Malaprade (11) that when thymolphthalein is used as an indicator (pH 9.3 to 10.5)  $\text{HIO}_4$  (which he found may exist as  $\text{H}_3\text{IO}_5$  or  $\text{H}_5\text{IO}_6$ ) acts as a bivalent acid with the use of 2 equivalents of alkali per mole of  $\text{HIO}_4$ , whereas  $\text{HIO}_3$  acts as a monovalent acid, 1 equivalent of alkali being used per mole of  $\text{HIO}_3$ . Therefore, when the reaction of  $\text{HIO}_4 \rightarrow \text{HIO}_3$  takes place, there is a decrease in the titratable acidity of the solution, this decrease being of 1 equivalent of acid per mole of  $\text{HIO}_4$  that changes to  $\text{HIO}_3$ . When, in the course of the reaction between a polyol and  $\text{HIO}_4$ , only  $\text{HCOOH}$  is formed, as is the case with inositol, the titratable acidity of the solution does not change because for each mole of  $\text{HIO}_4$  that becomes  $\text{HIO}_3$  1 mole of  $\text{HCOOH}$  is formed. When the reaction of  $\text{HIO}_4$  with polyols results in formation of aldehydes only, there is a decrease in the titratable acidity of the solution of 1 acid equivalent for each mole of  $\text{HIO}_4$  that becomes  $\text{HIO}_3$ . Therefore, when an unknown polyol reacts with  $\text{HIO}_4$ , it is possible to know how much formic acid has been produced if the initial amount of  $\text{HIO}_4$  present, the initial titratable acidity, the concentration of  $\text{HIO}_4$  at the end of the reaction, and the titratable acidity at the end of the reaction are known. The method that has been developed, and which is described in detail in the experimental part, provides us with this information. By this method it has been found that 1 mole of inositol diphosphate isolated among the cleavage





products of diphosphoinositide uses 2 moles of  $\text{HIO}_4$ , with production of 1 mole of  $\text{HCOOH}$ . This conclusively proves that the inositol diphosphate constituent of diphosphoinositide is inositol metadiphosphate.

Essentially no other progress has been made towards establishing the

structure of diphosphoinositide. Attempts to isolate glycerophosphoric acid have failed. This does not necessarily indicate that glycerol is not esterified to a phosphoryl radical, because, if glycerol is esterified to phosphoric acid by the least strong ester linkage in a phosphoric acid diester, this linkage may well undergo hydrolysis quite readily.

The claim has been made by Woolley (6) that the inositol-containing phosphatide from brain was closely related to, or identical with soy bean lipositol. The basis for this claim was a statement to the effect that he had succeeded in separating from brain lipides a fraction which contained carbohydrate (as galactose) and inositol in a 1:1 ratio and from which tartaric acid had been isolated after hydrolysis. No detailed evidence was given. Clearly, diphosphoinositide is quite different in chemical structure from lipositol. There are two possible explanations for Woolley's observation: One is the presence in brain of an inositol-containing phosphatide other than diphosphoinositide. While diphosphoinositide accounts for all of the inositol present in cephalin, no search has been made for inositol-containing lipides in other brain lipid fractions. The second possibility is that Woolley may have been dealing with diphosphoinositide grossly contaminated with carbohydrate-containing lipides. This would explain a carbohydrate to inositol ratio of 1. As for the isolation of tartaric acid the large amount of impurities found in crude diphosphoinositide makes it possible that tartaric acid could be present as a non-lipide contaminant.

#### EXPERIMENTAL

*Analytical Methods*—Most of the methods used have been enumerated elsewhere (2, 3). Carbohydrate has been estimated by the method of Sørensen and Haugaard (12), modified to suit the particular features of lipid material.  $\text{NH}_2\text{-N}$  analyses on acid hydrolysates have been carried out as described elsewhere (1). Carbon estimations on diphosphoinositide by dry combustion have consistently yielded lower results than those obtained by the manometric wet combustion method (13). Therefore, data by the latter method are given in the case of diphosphoinositide.

#### *Isolation of Inositol Diphosphate from Inositol Phosphatide*

The description of the procedure to isolate inositol metadiphosphate from inositol phosphatide is given here, because this isolation was the first step in the separation of diphosphoinositide from inositol phosphatide.

5.3 gm. of inositol phosphatide (2) were emulsified in 150 cc. of water and to the chilled emulsion were added 50 cc. of concentrated HCl. The precipitate thus formed was separated by centrifugation, washed twice with 100 cc. of 3 N HCl each time, and then hydrolyzed with boiling 6 N

HCl for 25 minutes. After cooling, the hydrolysate was filtered and the filtrate evaporated to dryness in a vacuum. The residue was dissolved in water and the solution was decolorized with charcoal. The water-clear filtrate was concentrated to a volume of 3 cc. and treated with 35 cc. of ethyl alcohol. On standing overnight in the ice box, the solution became cloudy. By centrifugation a liquid underlying layer of alcohol-insoluble material separated. The supernatant was decanted and discarded and the alcohol-insoluble material was dried and redissolved in 2.5 cc. of water. This solution was treated with 40 cc. of alcohol and let stand overnight in the ice box. A precipitate was obtained which, after drying, weighed 550 mg. This material was found to contain 3.74 per cent glycerol, 3.43 per cent  $\text{NH}_2\text{-N}$ , 10.5 per cent P, and <0.1 per cent inorganic P. It was dissolved in water and the solution was treated with neutral lead acetate. The insoluble lead salts that were formed were collected by centrifugation, washed three times with water, and then suspended in water and decomposed with  $\text{H}_2\text{S}$ , the lead sulfide thus formed being removed by filtration. The filtrate was dried. The dry residue weighed 230 mg. It was found to contain 1.9 per cent glycerol, <0.15 per cent  $\text{NH}_2\text{-N}$ , 16.5 per cent P, and <0.05 per cent inorganic P. This material was dissolved in 1.2 cc. of water, and the solution treated with 10 cc. of alcohol and let stand overnight in the ice box. A precipitate was formed which, after drying at  $140^\circ$  in a vacuum, weighed 210 mg.

The material obtained was a white powder which was not hygroscopic. The results of analysis proved it to have the composition of inositol diphosphate (Table I). Allowing for aliquots taken for analyses in the course of the procedure, the inositol in the pure inositol diphosphate isolated accounted for 40 per cent of the inositol in the starting material. Approximately the same yield was obtained in the case of the two other isolations carried out.

#### *Preparation of Brain Diphosphoinositide*

The preparation of inositol phosphatide from brain cephalin, and the separation from it of crude diphosphoinositide is described in an accompanying paper (2). This crude inositol phosphatide is obtained in yields of about 1 gm. per kilo of fresh tissue. It is grossly contaminated with water-soluble impurities, mainly inorganic phosphates. Its composition varies slightly from one preparation to another. Analytical results for a typical preparation are the following: C 31.5 per cent, N 0.79 per cent, P 11.08 per cent, inorganic P 6.10 per cent, carboxyl N 0.10 per cent, carbohydrate (as galactose) 1.7 per cent, glycerol 4.90 per cent, K 8.05 per cent, Na 3.25 per cent, Ca 0.30 per cent, Mg 1.30 per cent.

The crude material is purified by dialysis against distilled water in the

cold. After 4 days, dialysis appears to be completed, the composition of the undialyzable material remaining constant on further dialysis. At the end of the dialysis period, the undialyzable material is lyophilized.

On analysis, it is found to contain 46.0 per cent C, 7.3 per cent H, from 0.4 to 0.6 per cent N, from 0.1 to 0.4 per cent  $\text{NH}_2\text{-N}$  (after acid hydrolysis), between 7.0 and 7.3 per cent P, <0.05 per cent inorganic P, 9.0 per cent glycerol, between 0.4 and 1.8 per cent carbohydrate (as galactose), <0.02 per cent carboxyl N, 2.88 per cent Mg (average), 0.64 per cent Ca (average), and <0.01 per cent K and Na. Iodine number, 28.0. The (equivalents of base)/(atoms of P) ratio is 1.05 to 1.14. Inositol by bioassay (after 48 hours hydrolysis by boiling 6 N HCl) is 21.0 per cent. The difference in P

TABLE I

*Analysis of Inositol Diphosphate Isolated from Inositol Phosphatide and from Diposphoinositide*

Component	Inositol diphosphate from inositol phosphatide*	Inositol diphosphate from diposphoinositide†	Calculated for inositol diphosphate, $\text{C}_6\text{H}_{10}\text{O}_4(\text{H}_2\text{PO}_4)_2$ ‡
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
C.....	20.99	21.1	21.15
H.....	4.18	4.13	4.15
P.....	18.02	18.15	18.24
Inositol.....	54.00§	52.2	53.00
Inorganic P.....	<0.1	<0.05	
$\text{NH}_2\text{-N}$ .....	<0.02	<0.02	
Glycerol.....	<0.2	<0.2	

\* Neutral equivalent, 85.3.

† Neutral equivalent, 85.2.

‡ Calculated neutral equivalent, 85.0.

§ Determined by bioassay by Dr. D. W. Woolley.

|| By isolation of pure inositol.

content of different preparations appears to depend on the amount of carbohydrate-containing lipides present as contaminants, since the concentration of P and of carbohydrate in our six different preparations ranged in inverse order.

The material is a powder, which is only soluble in wet chloroform. The yield obtained is about half the amount of crude diposphoinositide, and represents between 0.5 and 0.6 gm. per kilo of fresh tissue.

*Estimation of Fatty Acids*—Fatty acids were estimated by the method used for estimation of fatty acids in phosphatidyl serine (3). Saponification was found to be complete after 4 hours. 210 mg. of one preparation yielded 112 mg. of a crystalline material; i.e., 53.2 per cent of starting material. Neutral equivalent, 400 (moles of fatty acids)/(atoms of P)

= 0.542. Other preparations analyzed have given neutral equivalent values as low as 376. The (moles of fatty acid)/(atoms of P) ratio is generally slightly above 0.5. It is likely that fatty acids are contributed by cerebrosides present in the preparations as contaminants.

*Acid Hydrolysis of Diphosphoinositide*—Weighed amounts of diphosphoinositide were treated with 6 N HCl in the boiling water bath for different lengths of time. After cooling, the hydrolysate was shaken with an equal volume of chloroform. The chloroform and aqueous phases were collected separately and analyzed for total P and inorganic P.

At zero time all the P was found in the chloroform phase. At 10 minutes time and subsequently, all the P was found in the 6 N HCl phase. At 10, 20, and 40 minutes, 2½, 5½, 17, and 30 hours, the percentage of P as inorganic P in the 6 N HCl phase was respectively 4, 6, 10, 30, 50, 90, and 100.

*Isolation of Inositol from Diphosphoinositide*—1 gm. of diphosphoinositide was suspended in 120 cc. of water and 40 cc. of concentrated HCl were added to the suspension. After stirring thoroughly, the suspended material was collected by centrifugation. The supernatant was discarded. On addition of 120 cc. of water, the residue went readily into emulsion. The emulsified material was precipitated by addition of 40 cc. of concentrated HCl. The precipitate was collected by centrifugation, transferred to a boiling flask, and hydrolyzed by boiling 6 N HCl under a reflux for 48 hours. After cooling, the hydrolysate was filtered and the filtrate evaporated to dryness in a vacuum. The residue was dissolved in 30 cc. of water and 0.5 cc. of concentrated acetic acid and 2 gm. of Ag<sub>2</sub>O added to it. The mixture was centrifuged, the supernatant solution collected, and the insoluble silver salts washed twice with small amounts of 1 per cent aqueous acetic acid solution. The washings were combined with the supernatant solution, and the combined solution treated with H<sub>2</sub>S. The filtrate from the silver sulfide was evaporated to dryness in a vacuum. The residue was dissolved in 2 cc. of water and 40 cc. of ethyl alcohol added to the solution. On standing overnight in the ice box, crystals formed, which were collected by centrifugation. After drying at 100° in a vacuum, the crystals weighed 170 mg. Since they were found to contain 1.88 per cent ash, they were recrystallized twice from solution in 2 cc. of 0.1 N HCl by addition of 40 cc. of ethyl alcohol. The twice recrystallized crystals (140 mg.) proved to be analytically pure inositol. The analytical results were as follows: C 39.88 per cent, H 6.64 per cent, ash 0.3 per cent, m.p. 218.0° (uncorrected); mixed melting point with inositol, 218.0°. The theory for inositol is C 39.98 per cent and H 6.67 per cent. Allowing for aliquots taken for ashing, the amount obtained accounted for 16 per cent of the weight of starting diphosphoinositide.

*Isolation of Inositol Diphosphate from Diphosphoinositide*—1233 mg. of diphosphoinositide (containing 7 per cent P) were suspended in 50 cc. of 0.5 N HCl and dialyzed against distilled water for 3 days, the water being changed twice a day. The diphosphoinositide went into solution inside the dialysis bag. To this solution was added an equal volume of concentrated HCl and the mixture was heated in a boiling water bath for 15 minutes. The resulting hydrolysate was cooled and extracted with an equal volume of chloroform. The acid supernatant was collected and dried by evaporation in a vacuum. The residue was dissolved in 40 cc. of H<sub>2</sub>O and 6 cc. of 25 per cent aqueous neutral lead acetate solution were added to it. After standing for 1 hour in the ice box, the insoluble lead salts were collected by centrifugation and washed twice, each time with 20 cc. of ice-cold 3 per cent neutral lead acetate aqueous solution. The precipitate was suspended in 30 cc. of water and the lead was removed by treatment with H<sub>2</sub>S. The filtrate from the lead sulfide was dried by evaporation in a vacuum. The residue weighed 400 mg. and contained 19 per cent P, 0.6 per cent inorganic P, and <1 per cent glycerol. It was dissolved in 2 cc. of water, a slight amount of insoluble material was eliminated by centrifugation, and the clear supernatant solution was treated with 20 cc. of alcohol. After standing overnight in the ice box, the precipitate that formed was collected and dried. It weighed 333 mg. The results of analysis are given in Table I. They showed that the material was inositol diphosphate. The crude inositol diphosphate accounted for 83.5 per cent of the P present in the mother substance. The analytically pure inositol diphosphate accounted for 69.7 per cent of the P present in the mother substance.

*Chemical Structure of Inositol Metadiphosphate Isolated from  
Diphosphoinositide*

The chemical structure of this compound has been established (1) by its chemical analysis, (2) by isolation from it of inositol, (3) by the results of its titration, which show that each phosphoryl radical has two free acid groups, and (4) by the study of the products of its reaction with HIO<sub>4</sub>, which show that the two phosphoryl radicals in the inositol diphosphate molecule are in the meta position.

*Isolation of Inositol from Inositol Diphosphate*—134 mg. of inositol diphosphate were treated with 20 cc. of 6 N HCl in the boiling water bath for 40 hours. After cooling, the solution was taken to dryness by evaporation in a vacuum. The residue was dissolved in 10 cc. of water, 0.1 cc. of concentrated acetic acid was added to it, and the solution was treated with 300 mg. of Ag<sub>2</sub>O. The insoluble material was collected by centrifugation. The supernatant solution was decanted, and the precipitate was

washed twice with 1 cc. of 1 per cent aqueous acetic acid each time, after which the washings were combined with the supernatant and the combined solutions were treated with  $\text{H}_2\text{S}$ . The filtrate from the silver sulfide was dried by evaporation in a vacuum. The residue was taken up in 0.5 cc. of water and treated with 10 cc. of ethyl alcohol. After standing overnight in the ice box, the crystalline precipitate that formed was collected and dried. It weighed 70 mg. and on analysis proved to be analytically pure inositol. It accounted for 52.2 per cent of the weight of the original material; *i.e.*, 98.4 per cent of the inositol supposed to be present in inositol diphosphate. The analytical results were as follows:

$\text{C}_6\text{H}_{12}\text{O}_6$ . Calculated.	C 39.98, H 6.67
Found (corrected for 0.68% ash).	“ 39.93, “ 6.69
M.p. (uncorrected) 217.8°; mixed m.p. with inositol 217.6°	

*Reaction of Inositol Diphosphate from Diphosphoinositide with  $\text{HIO}_4$ ;*  
*Method*—A solution of known concentration of inositol diphosphate, neutralized with alkali to the turning point of thymolphthalein, was mixed with a solution of  $\text{HIO}_4$  of such strength that the concentration of  $\text{HIO}_4$  in the final solution was 4-fold the molar concentration of inositol diphosphate. The progress of the reaction was followed by taking aliquots of the solution after different lengths of time. Each aliquot was handled as follows: Thymolphthalein indicator was added and the acidity was titrated with standard  $\text{NaOH}$ . Then, the pH was brought back to 8.2 by addition of saturated solution of sodium bicarbonate. Excess  $\text{KI}$  solution was then added and the iodine liberated was titrated with standard arsenious acid solution as a measure of the  $\text{HIO}_4$ . At each experimental point on the time curve, the concentration of remaining  $\text{HIO}_4$  was ascertained by the titration with arsenious acid and the concentration of  $\text{HIO}_3$  present was given by the difference between the original known concentration of  $\text{HIO}_4$  and the concentration of  $\text{HIO}_4$  found. From these two values the titratable acidity due to  $\text{HIO}_4$  and  $\text{HIO}_3$  present can be computed, the former acid acting as bivalent and the latter as monovalent when titrated to the turning point of thymolphthalein. Therefore, any titratable acidity in excess of the value computed for  $\text{HIO}_4$  and  $\text{HIO}_3$  present was due to formic acid.

The following actual experimental conditions have been selected: 0.2 mm of inositol diphosphate (68 mg.) was placed in a 25 cc. volumetric flask, dissolved in 3 cc. of water, 3 drops of thymolphthalein indicator solution were added, and the solution was brought to the turning point of thymolphthalein by the addition of 0.1 N  $\text{NaOH}$ . 0.8 mm of  $\text{HIO}_4$  solution (4 cc. of 0.2 M  $\text{HIO}_4$  solution) was added to the contents of the flask and the solution was made up to the mark with distilled water. At 15 and 30 minutes,

and 2, 8, 16, 24, and 48 hours, duplicate 1 cc. aliquots were taken and titrated with 0.04 N NaOH. To the titrated aliquot were then added in succession 1 cc. of saturated sodium bicarbonate solution and, after mixing, 0.5 cc. of 20 per cent aqueous KI solution. The iodine liberated was then titrated with 0.04 N arsenious acid solution. These conditions have been selected so that the error in both titrations becomes <1 per cent of the values obtained.

At room temperature the reaction is complete in 24 hours, the same value being obtained at 24 and 48 hours. A blank was run in which distilled water was used instead of the solution of inositol diphosphate. It has been consistently found that  $\text{HIO}_4$  is stable under the conditions and for the length of time of the method.

Repeated experiments on two different samples of inositol diphosphate have shown that 0.2 mm of inositol diphosphate uses 0.4 mm of  $\text{HIO}_4$  and produces between 0.18 and 0.19 mm of formic acid. The fact that the formic acid production falls short of the calculated amount for inositol metadiphosphate (which is 1 mole per mole of inositol diphosphate) is consistent with the behavior of inositol on reacting with  $\text{HIO}_4$  (10). In this reaction the production of formic acid also runs between 90 and 95 per cent of the theoretical.

A consideration of the ratio of the moles of  $\text{HCOOH}$  produced to the moles of  $\text{HIO}_4$  used at the different experimental points shows that in the early stages of the reaction between inositol diphosphate and  $\text{HIO}_4$  the amount of formic acid produced lags behind the amount of  $\text{HIO}_4$  used. This is also the case with inositol (10) and is probably due to intermediate reactions.

Of course, any hydrolysis of inositol diphosphate would invalidate the value of the results reported here. Therefore, at the end of 48 hours the remaining reaction mixture was tested for inorganic phosphate. In all cases a negative result was obtained and the conditions used established that <0.1 per cent of the inositol diphosphate could have been hydrolyzed.

The reaction between  $\text{HIO}_4$  and inositol diphosphate has been studied with excess of  $\text{HIO}_4$  varying between 50 and 500 per cent. In all cases the same amounts of  $\text{HIO}_4$  have been used and the same amount of formic acid produced.

#### *Preparation of Base-Free Diposphoinositide*

2.5 gm. of diposphoinositide were suspended in 40 cc. of  $\text{H}_2\text{O}$  and 4 cc. of concentrated  $\text{HCl}$  were added dropwise to the chilled suspension. The mixture was stirred for 30 minutes in the cold. On centrifugation a slightly opalescent supernatant was obtained. It was discarded. The precipitate was emulsified in 40 cc. of  $\text{H}_2\text{O}$ . The emulsion was transferred to a dialy-



sis bag and dialyzed in a shaking machine three times in succession against 2 liters of cold distilled water each time, for a total shaking time of 6 hours. The emulsion was then lyophilized. 2.1 gm. of a whitish powder were obtained. Total P 7.51 per cent, inorganic P <0.1 per cent. This material was found to be free of calcium and magnesium.

The base-free diphosphoinositide went easily into emulsion in water. It was soluble in wet chloroform and wet ethyl ether. On titration to the turning point of phenolphthalein, base-free diphosphoinositide used 1 equivalent of NaOH for each atom-gm. of P present, thereby showing that the phosphoric acid in its molecule was in the form of a diester with one acid group remaining free. On treatment by acid, as described above, diphosphoinositide appeared to be partly destroyed. This could be shown by dissolving 1 part of base-free diphosphoinositide in 30 parts of wet ethyl ether and adding to the solution 150 parts of acetone. On standing overnight in the cold, a light precipitate separated. After 1 hour's centrifugation a fairly clear supernatant was obtained. After drying the supernatant and precipitate separately, it was found that the acetone-insoluble fraction contained 8.8 per cent P, while the acetone-soluble material contained 4.1 per cent P. The acetone-soluble fraction, when again submitted to the ether-acetone treatment described above, yielded an acetone-insoluble and an acetone-soluble fraction. The former contained 8.8 per cent P and the latter 1.86 per cent. This fraction was again treated with ether-acetone and an acetone-soluble subfraction was obtained which contained 1.00 per cent P. This subfraction, which represented about one-sixth of the weight of the original base-free diphosphoinositide, was found to contain 9 per cent glycerol, 50 per cent fatty acids, and 4 per cent carbohydrate (as galactose). The fatty acids were combined, presumably with glycerol. Therefore, the substance appeared to be a mixture of carbohydrate-containing lipides, mono- and diglycerides, and unchanged diphosphoinositide. This observation suggests that fatty acids in the diphosphoinositide molecule are esterified with a glycerol radical.

#### *Preparation of K Salt of Diphosphoinositide*

The potassium salt of diphosphoinositide can be prepared by treatment of the Ca-Mg diphosphoinositide with potassium oxalate, as follows: 1 gm. of diphosphoinositide is placed in a 50 cc. centrifuge tube and 40 cc. of a 20 per cent solution of potassium oxalate added to it. On stirring, the material goes rapidly into emulsion. The emulsion is cloudy owing to the presence of calcium oxalate. The emulsion is brought to pH 8 by dropwise addition of N potassium hydroxide. The mixture is allowed to stand overnight in the ice box to insure complete precipitation of the insoluble oxalates. The solution is then centrifuged for 1 hour at 2500 R.P.M. The

supernatant is transferred to another centrifuge tube and centrifuged for 30 minutes. If the second centrifugation yields a precipitate, the supernatant is transferred to another tube and centrifuged again for 30 minutes. Centrifugation is repeated until no precipitate collects. Usually, two centrifugations are sufficient. The supernatant is then transferred to a dialysis bag and dialyzed against distilled water for several days with frequent changes of the outside water. The material is finally lyophilized. The substance thus obtained gives an (equivalents of K)/(atoms of P) ratio of 1.00. If this ratio is higher than 1, the material still contains potassium oxalate and should be dialyzed again.

The potassium salt of diphosphoinositide is insoluble in ethyl alcohol, methyl alcohol, acetone, petroleum ether, and chloroform. It goes readily into emulsion in water, a 20 per cent emulsion being perfectly stable to centrifugation at 12,000 R.P.M. While, as stated above, the compound appears to be insoluble in all the organic solvents that have been used, it is possible to dilute a 5 per cent aqueous emulsion with methyl alcohol or methyl alcohol-chloroform (1:2) mixture without any precipitation occurring. This can be made use of to obtain a solution of the material in an organic solvent.

#### SUMMARY

1. Diphosphoinositide, a new brain phosphatide, has been separated from brain cephalin. This phosphatide accounts for all of the inositol present in brain cephalin. The constituents of the new phosphatide appear to be inositol metadiphosphate, glycerol, and fatty acids in equimolar proportions.

2. Inositol metadiphosphate, which has been isolated among the products of short time acid hydrolysis of diphosphoinositide, has been identified by its elementary composition, by titration with alkali, by isolation from it of inositol, and by the study of the products of its reaction with  $\text{HIO}_4$ . On reacting with  $\text{HIO}_4$ , each mole of inositol diphosphate has been found to use 2 moles of  $\text{HIO}_4$  and to produce 1 mole of  $\text{HCOOH}$ . This is consistent with a meta position of the two phosphoryl radicals on the inositol molecule.

3. Besides the constituents enumerated above, diphosphoinositide contains as contaminants carbohydrate-containing lipides. Nitrogen present in diphosphoinositide preparations also appears to be a contaminant.

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# RATE OF ELIMINATION OF RADIOACTIVE CARBON ADMINISTERED AS CARBONATE FROM THE TISSUES AND TISSUE COMPONENTS OF MATURE AND GROWING RATS\*

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(Received for publication, August 27, 1948)

The widespread incorporation of  $C^{14}$  from carbonate in several tissues and tissue components of unfasted mature rats was described in an earlier report (1). The present paper is concerned with the rate at which the incorporated  $C^{14}$  is eliminated from the tissues of mature and growing rats. These problems are of particular importance for evaluating the potential health hazards of the long lived radiocarbon.

## EXPERIMENTAL

Eight mature and six young Sprague-Dawley male rats were employed. The diet given previous to and during the experimental period consisted of a commercial dog biscuit and water, both supplied *ad libitum*. The animals were given  $C^{14}$ -labeled sodium carbonate by intraperitoneal injections of a solution made isotonic with sodium chloride. Details concerning the animals and the dosage of  $C^{14}$  which they received are given in Table I. Pairs of the animals were sacrificed at intervals of time after the administration of the last dose of labeled sodium carbonate, as indicated in Table I. Shortly before being sacrificed each animal was placed in a closed flask through which  $CO_2$ -free air was drawn into saturated barium hydroxide. The barium carbonate which precipitated was collected and prepared for measurement of its  $C^{14}$  activity as previously described (2). The animals were anesthetized by an intraperitoneal injection of sodium pentobarbital and sacrificed by slitting their throats, the blood being collected in centrifuge tubes. All tissues and tissue components to be examined were prepared and purified for total carbon analysis and  $C^{14}$  assay by methods previously described (1).

Measurements of radioactivity were made by counting solid samples of  $BaC^*O_3$  (2) ( $C^*$  indicates carbon labeled with  $C^{14}$ ) obtained from the oxidation of the sample (3) or by direct counts of "infinitely thick" samples of the dried and pulverized materials (1). Thin (2.0 to 2.5 mg. per sq. cm.) end mica window, Geiger tubes were used for the detection of the radiation.

\* This study was supported by a grant from the Research Grants Division of the United States Public Health Service. The radiocarbon used in this investigation was obtained on allocation from the United States Atomic Energy Commission.

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Corresponding materials from the several animals were prepared in the same manner and counted with the same counter and scaler apparatus. The total carbon content of the materials which were counted directly was determined on an aliquot by the method of Lindenbaum, Schubert, and Armstrong (3).

TABLE I

*Weights and Dosage Data of Rats Receiving Radiocarbon As Sodium Carbonate*

The animals were given by intraperitoneal injection a 0.002 M  $\text{Na}_2\text{C}^*\text{O}_3$  solution made isotonic with NaCl. All animals received three injections at 2 hour intervals. Rats 1a to 6a received a total of 3.0 cc. of solution containing 0.13 millicurie of  $\text{C}^{14}$ . Rats 1 to 8 received 5.3 to 7.2 cc. of solution containing 0.19 to 0.25-millicurie of  $\text{C}^{14}$ .

Rat No.	Time interval between last injection and sacrifice	Weights		Rat No.	Time interval between last injection and sacrifice	Weights	
		When injected	When sacrificed			When injected	When sacrificed
	<i>days</i>	<i>gm.</i>	<i>gm.</i>		<i>days</i>	<i>gm.</i>	<i>gm.</i>
1a	8	44.5	91	1	7.9	525	508
2a	8	45	91	2	7.9	495	488
3a	15	45	139	3	15	512	502
4a	15	45	139	4	15	532	520
5a	22	45	161	5	20	497	520
6a	22	45	171	6	20	472	480
				7	30	500	515
				8	30	500	525

## RESULTS AND DISCUSSION

The changes in the  $\text{C}^{14}$  activity in the tissues and tissue components following the administration of  $\text{Na}_2\text{C}^*\text{O}_3$  are given in Tables II and III. The results are expressed as specific activity (the specific activity = the percentage of the administered dose of  $\text{C}^{14}$  per mg. of carbon).

The results show that by the 8th day after the administration of  $\text{C}^{14}$  the  $\text{C}^{14}$ -specific activities of all tissues of the growing animals, except the serum proteins, were much higher than the corresponding tissues of the mature animals. The amounts and rate of change of  $\text{C}^{14}$  concentration in the serum proteins were about the same in the two groups of animals. It can be assumed that the higher concentration of  $\text{C}^{14}$  in the tissues of the growing animals was a result not only of the reconstitution of tissues existing at the time of the administration of the  $\text{C}^{14}$  but also was due to the formation by growth of new tissues from a milieu containing  $\text{C}^{14}$ . In the case of the mature animals the  $\text{C}^{14}$  was incorporated only by the process of reconstitution of existing tissues. The results obtained at the 30th day, in the case of the mature rats, indicated that the residual  $\text{C}^{14}$  activity in the soft tissues was still decreasing but at a much reduced rate. For

TABLE II

*Change in C<sup>14</sup> Concentration in Tissues and Tissue Components of Growing Rats with Time\**

Sample	Specific activity† × 10 <sup>6</sup> of administered C <sup>14</sup> found after					
	8 days		15 days		22 days	
	Rat 1a	Rat 2a	Rat 3a	Rat 4a	Rat 5a	Rat 6a
Bone, intact	118	113	36.6	46.8	27.8	31.8
“ inorganic fraction	±1.5	±1.8	±1.1	±1.3	±0.9	±0.9
“ protein	2120	2060	675	707	462	473
	±14	±16	±8.2	±7.1	±5.2	±6.2
	39.5	42.9	18.2	22.4	16.0	18.9
	±0.6	±0.8	±0.5	±0.6	±0.4	±0.5
Teeth, intact incisors‡	194 ± 2.9		110 ± 2.3		75.3 ± 2.2	
“ “ molars‡	169 ± 2.8		101 ± 2.4		107 ± 2.2	
Muscle, protein	40.2	49.4	22.3	18.9	11.3	13.4
	±0.7	±0.8	±0.5	±0.5	±0.4	±0.4
Liver, “	106	162	68.2	40.7	11.1	8.4
	±1.2	±1.5	±0.8	±0.7	±0.3	±0.3
Brain, “			27.0	25.4	24.2	23.2
			±0.6	±0.6	±0.5	±0.6
Serum albumin	30.7 ± 5.8		6.5 ± 1.8		2.8 ± 1.2	
“ globulin	13.7 ± 0.7		7.9 ± 1.9		0.85 ± 0.5	
Red blood cells	36.5 ± 0.6		25.2 ± 0.5		12.9 ± 0.4	
Residue§	45.0	30.8	33.7	12.7	8.4	8.0
	±2.4	±1.9	±1.2	±0.9	±0.7	±0.6
Respiratory CO <sub>2</sub>	13.4	10.6	2.3	4.4	2.1	0
	±1.4	±1.5	±1.2	±1.3	±1.3	±1.3

\* See Table I for weights and injection data.

† The deviations of the results given are derived from the statistical errors of counting and are equal to the square root of the sum of the squares of the standard deviations of the sample and background counts.

‡ The values of the specific activities of these tissues were obtained from samples which were pooled from a given pair of rats.

§ Includes perf without bones, teeth, liver, and blood.

example, the biological half life of the C<sup>14</sup> in the liver protein of the mature animals, estimated from the changes in specific activity between the 20th and the 30th day, is about 37 days.<sup>1</sup>

<sup>1</sup> The biological half lives of C<sup>14</sup> referred to in this paper were estimated from a plot of the data in Tables II and III on semilogarithmic paper in which specific activities were plotted on the logarithmic axis and time on the regular axis. In this way curves analogous to radioactive decay curves were obtained. It is to be understood that the biological half lives of C<sup>14</sup> reported here apply only to the particular experimental conditions given in this paper. Variations might be expected from these results, particularly in the case of the soft tissues, depending, for example, upon the nature of the compound containing the C<sup>14</sup> administered to the animals.

The more active metabolic processes in the young rats are exemplified in the results obtained with the muscle protein. Between the 8th and the 22nd day the  $C^{14}$  activity of the muscle protein decreased about 4-fold in

TABLE III

*Change in  $C^{14}$  Concentration in Tissues and Tissue Components of Mature Rats with Time\**

Sample	Specific activity† $\times 10^6$ of administered $C^{14}$ found after							
	7.9 days		15.0 days		20.0 days		30.0 days	
	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Rat 7	Rat 8
Bone, intact	9.2	9.1	5.9	5.3	4.9	4.8	4.0	3.8
	$\pm 0.6$	$\pm 0.6$	$\pm 0.4$	$\pm 0.4$	$\pm 0.6$	$\pm 0.4$	$\pm 0.5$	$\pm 0.4$
“ inorganic fraction	49.6	44.4	41.4	40.2	37.0	31.5	30.4	36.0
	$\pm 1.2$	$\pm 1.2$	$\pm 1.3$	$\pm 1.2$	$\pm 1.7$	$\pm 1.3$	$\pm 1.0$	$\pm 1.0$
“ protein	3.0	4.4	1.6	1.1	1.2	1.3	0.85	1.1
	$\pm 0.2$	$\pm 0.2$	$\pm 0.1$	$\pm 0.1$	$\pm 0.2$	$\pm 0.1$	$\pm 0.1$	$\pm 0.1$
Teeth, intact incisors	31.2	32.9	26.7	28.9	34.9	30.4	20.7	20.5
	$\pm 1.0$	$\pm 1.1$	$\pm 1.1$	$\pm 1.0$	$\pm 1.2$	$\pm 0.95$	$\pm 0.8$	$\pm 0.8$
“ inorganic fraction of incisors‡	324 $\pm$ 5.0		319 $\pm$ 5.0		350 $\pm$ 6.0		275 $\pm$ 3.6	
Teeth, intact molars‡	7.2 $\pm$ 0.6		4.2 $\pm$ 0.5		3.9 $\pm$ 0.6		0.54 $\pm$ 0.9	
Liver, protein	18.8	19.7	6.6	5.0	3.9	4.3	2.0	4.8
	$\pm 0.3$	$\pm 0.3$	$\pm 0.2$	$\pm 0.2$	$\pm 0.2$	$\pm 0.2$	$\pm 0.2$	$\pm 0.2$
“ glycogen	13.3	10.7	1.2	1.1	3.5	1.7	1.75	
	$\pm 0.3$	$\pm 0.2$	$\pm 0.1$	$\pm 0.2$	$\pm 0.2$	$\pm 0.1$	$\pm 0.1$	
Serum, globulin	10.6	17.8	3.4	3.3	1.9	2.2	0.80	0.89
	$\pm 0.7$	$\pm 0.7$	$\pm 0.2$	$\pm 0.2$	$\pm 0.2$	$\pm 0.2$	$\pm 0.4$	$\pm 0.2$
“ albumin	17.9	38.0	5.4	4.3	1.8	2.5	2.7	1.6
	$\pm 0.9$	$\pm 1.2$	$\pm 0.2$	$\pm 0.2$	$\pm 0.3$	$\pm 0.2$	$\pm 1.0$	$\pm 0.5$
Muscle, protein	4.0	7.0	4.4	5.0	5.1	5.6	2.5	2.6
	$\pm 0.2$	$\pm 0.2$	$\pm 0.2$	$\pm 0.2$	$\pm 0.2$	$\pm 0.2$	$\pm 0.2$	$\pm 0.2$
Residue§	7.9	15.8	4.5	5.7	6.6	6.8	6.0	4.1
	$\pm 0.2$	$\pm 0.4$	$\pm 0.2$	$\pm 0.2$	$\pm 0.3$	$\pm 0.2$	$\pm 0.2$	$\pm 0.2$
Respiratory $CO_2$	7.0	8.4	4.3	4.4	3.8	2.3	4.4	2.4
	$\pm 1.0$	$\pm 0.9$	$\pm 0.9$	$\pm 0.9$	$\pm 0.6$	$\pm 0.5$	$\pm 0.6$	$\pm 0.6$

\* See Table I for data concerning weights and injection data.

† The deviations of the results given are derived from the statistical errors of counting and are equal to the square root of the sum of the squares of the standard deviations of the sample and background counts.

‡ The values of the specific activities of these tissues were obtained from samples which were pooled from a given pair of rats.

§ Includes pelt without bone, teeth, liver, and blood.

the young rats, but remained nearly unchanged in the mature animals from the 8th to the 20th day.

The  $C^{14}$  content of the skeleton decreased continuously. The biological half life of the  $C^{14}$  of whole bone and of the inorganic carbon in bone was



about 12 to 15 days in the growing animals and 30 to 40 days in the mature rats. This result is not in agreement with the preliminary data of Bloom, Curtis, and McLean (4). These workers found that radioautographs of

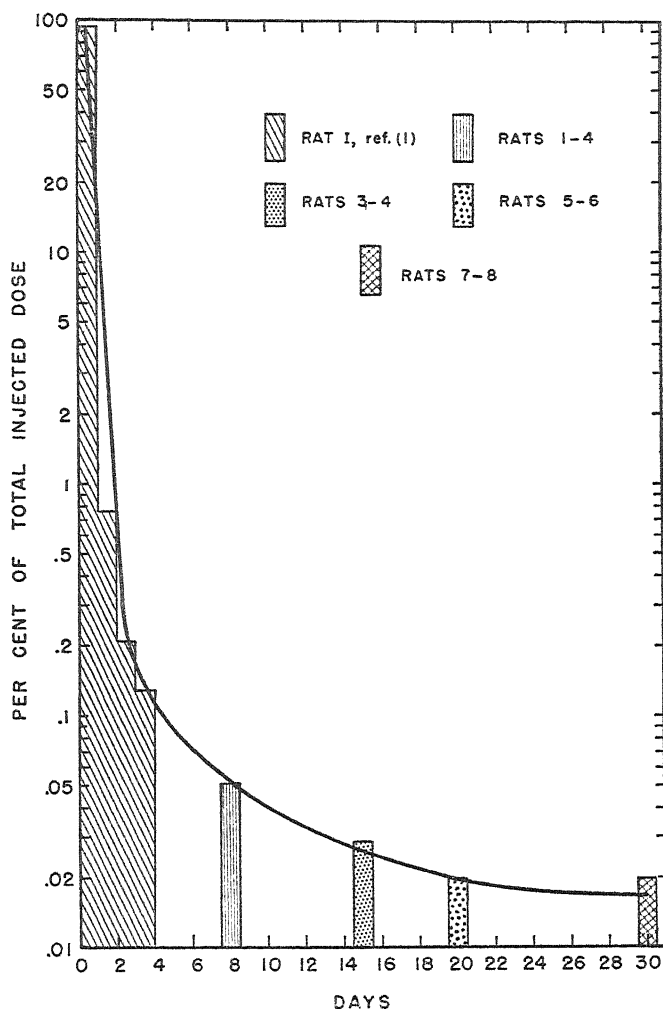


FIG. 1. Rate of excretion of  $C^{14}$  in the exhaled  $CO_2$  from mature rats following intraperitoneal injection of  $C^{14}$ -labeled sodium carbonate. The data for Rats 1 to 8 were calculated on the assumption that these rats exhaled 560 mm of  $CO_2$  in a 24 hour period (1).

bone indicated no decrease in the  $C^{14}$  content of bone in a period between 3 days and 16 weeks. Between the 8th and the 15th day the specific activity of the bone proteins in both groups of rats employed in the present

study decreased rapidly. Beyond the 15th day, however, the rate of decrease diminished considerably.

The difference in the uptake of  $C^{14}$  by growing and non-growing tissue is also exemplified by the results found with the molar and incisor teeth of the mature rats. The specific activity of the growing incisor teeth was at least 5 times that of the non-growing molar teeth. In the young rats in which both types of teeth were still growing there was little difference in the amounts of incorporation of  $C^{14}$ . Beyond the 15th day the biological half life of  $C^{14}$  in the incisor teeth of the mature rats was found to be about 36 days as compared to about 12 days in the young animals.

TABLE IV

*Per Cent of Total Injected Dose of  $C^{14}$  Administered As  $Na_2C^{14}O_3$  Remaining in Rats at Time of Sacrifice\**

Rat No.	Time elapsed after injection	$C^{14}$ retained	
			Average
	<i>days</i>		
1a	8	0.62	0.55
2a	8	0.47	
3a	15	0.53	0.42
4a	15	0.30	
5a	22	0.20	0.22
6a	22	0.23	
1	7.9	0.73	0.96
2	7.9	1.19	
3	15	0.47	0.50
4	15	0.52	
5	20	0.70	0.65
6	20	0.60	
7	30	0.48	0.50
8	30	0.51	

\* See Table I for weights and injection data.

The  $C^{14}$ -specific activity of the exhaled carbon dioxide of the growing animals was about 50 per cent greater than that of the mature animals on the 8th day but by the 15th day was, within experimental error, equal to that of the mature animals. It is of interest to combine the data for the expired carbon dioxide of the mature rats with similar data obtained in a previous study (1) with mature animals (Fig. 1).

As already noted, the specific activity of the carbon of the tissues of the young animals was generally much greater than that of corresponding tissues of the mature animals; nevertheless, the total  $C^{14}$  retention, expressed as a percentage of the total dose of  $C^{14}$ , was appreciably less in the case of the growing animals, as is shown in Table IV. This situation finds an obvious explanation in the fact that, although the specific retention of

C<sup>14</sup> by the growing animals was high, the total mass of tissues in their bodies was relatively small. Between the 15th and 30th days the total C<sup>14</sup> retained by the mature rats remained essentially unchanged in contrast to the marked decrease in over-all C<sup>14</sup> retention exhibited by the growing rats (Table IV). This result again exemplifies the more rapid reconstitution of the tissues of the growing animals in comparison to that of mature animals.

The results obtained with the brain protein and red blood cells of the young animals are presented as items of general interest. It is to be noted that there was no significant turnover of C<sup>14</sup> in brain protein after the radioisotope had become incorporated in this organ during its period of rapid growth.

#### SUMMARY

Eight mature rats and six growing rats were given intraperitoneal injections of C<sup>14</sup>-labeled sodium carbonate in order to study the change in C<sup>14</sup> concentration of the soft and calcified tissues and derived components with time. The rats were killed in pairs at different time intervals from 8 to 30 days after the last of the injections.

In general the specific activity of C<sup>14</sup> in the tissues and tissue components of the growing rats greatly exceeded those of the mature animals. The over-all retention of C<sup>14</sup>, however, was greater in the mature rats. In the latter practically no change in the over-all C<sup>14</sup> retention was observed after the 15th day, while an appreciable decrease in over-all retention of C<sup>14</sup> was observed in the growing animals.

Beyond the 8th day the biological half lives of the C<sup>14</sup> in intact and inorganic bone were 12 to 15 days in the growing rats and 30 to 40 days in the mature rat.

In the case of the serum proteins the specific activity of C<sup>14</sup> and the rates of decrease in C<sup>14</sup> content were about the same for the two groups of rats. The rate of decrease of C<sup>14</sup> in the soft tissues of the mature rats had decreased appreciably by the 20th day. No significant turnover of C<sup>14</sup> in the brain protein was observed in the young animals following its incorporation.

We wish to express our thanks to Mary L. Gouze, Carol Carlson, Mary L. Smersh, and Arthur Lindenbaum for their technical assistance.

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## THE AMINO ACID CONTENT OF SOME SCLEROPROTEINS

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(Received for publication, August 27, 1948)

Knowledge concerning the amino acid content of the scleroproteins is far from complete. The summary tables of Block and Bolling (1), Cohn and Edsall (2), Schmidt (3), and Hawk *et al.* (4) offer the best available data, but there is much disagreement. Lindley (5) and Geiger (6) have recently reported on the constitution of wool.

We are reporting on the content of fourteen amino acids in seven scleroproteins. In some instances, our values differ substantially from reports in the literature. In other instances, the content of certain amino acids is reported for the first time.

### EXPERIMENTAL

#### Methods

The samples were hydrolyzed and prepared for assay as described by Hier *et al.* (7). Amino acids were determined by the following microbiological procedures: methionine and threonine with *Streptococcus faecalis* and the medium of Stokes *et al.* (8); arginine and tyrosine with *Lactobacillus delbrueckii* and the above medium; lysine, histidine, and aspartic acid with *Leuconostoc mesenteroides* with Medium D of Dunn *et al.* (9); leucine, isoleucine, valine, phenylalanine, and glutamic acid with *Lactobacillus arabinosus* according to the method of Hier *et al.* (7); and proline by the method of Barton-Wright *et al.* (10). Cystine was determined by the chemical method of Kassell and Brand (11).

*Preparations Analyzed*—The nitrogen values are reported on a water- and ash-free basis.

*Achilles Tendon*—Bovine origin; trimmed from extraneous fat and muscle tissue, hashed, and dried *in vacuo*; total nitrogen, 16.9 per cent.

*Gelatin*—Wilson's pure food gelatin derived from pork skin; nitrogen content, 17.6 per cent.

*Yellow Tendon*—Bovine ligamentum nuchae, trimmed, hashed, and dried *in vacuo*; total nitrogen, 15.5 per cent.

*Elastin*—Prepared by the method of Richards and Gies (12) from yellow tendon; total nitrogen, 16.6 per cent.

*Wool*—Sheep wool defatted with benzene and dried *in vacuo*; total nitrogen, 16.2 per cent.

*Feathers*—Chicken feathers, washed with water and dried *in vacuo*, total nitrogen, 15.0 per cent.

*Hair*—Hog hair, washed with water and dried as above; total nitrogen, 16.6 per cent.

*Horn*—Cattle horn; washed with water and dried *in vacuo*, total nitrogen, 15.6 per cent.

### Results

Table I shows the results of the analyses of the preparations described above.

TABLE I  
*Amino Acid Content of Some Scleroproteins*

The values (in per cent) are on a water and ash-free basis.

Amino acid	Achilles tendon (1)	Gelatin* (2)	Yellow tendon (3)	Elastin (4)	Wool (5)	Feathers (6)	Hair (7)	Horn (8)
Arginine.....	8.0	8.0	3.1	1.1	10.6	7.5	10.9	10.7
Aspartic acid.....	6.9	6.7	2.9	0.6	7.2	7.0	8.0	7.7
Cystine.....	0.3	0.07	0.7	0.6	13.7	8.2	14.4	12.1†
Glutamic acid.....	10.4	11.5	5.0	3.3	15.6	9.7	17.9	13.8
Histidine.....	0.9	0.79	0.3	0.04	1.1	0.4	1.1	1.0
Isoleucine.....	1.9	1.4	4.3	3.4	4.5	6.0	4.7	4.3
Leucine.....	3.5	3.2	7.6	7.3	8.1	8.0	8.3	8.3
Lysine.....	3.5	4.1	1.3	0.5	3.3	1.3	3.8	3.6
Methionine.....	0.9	1.0	0.3	0.03	0.6	0.5	0.5	0.5
Phenylalanine.....	2.5	2.2	4.3	4.8	4.0	5.2	2.7	3.2
Proline.....	10.5	18.0	12.6	15.6	8.1	8.8	9.6	8.2
Threonine.....	2.5	1.9	1.5	1.1	6.7	4.4	6.3	6.1
Tyrosine.....	0.9	0.44	1.7	1.4	5.6	2.2	3.5	5.6
Valine.....	2.9	2.5	13.6	13.8	5.7	8.3	5.9	5.5
Nitrogen.....	16.9	17.6	15.5	16.6	16.2	15.0	16.6	15.6

\* A part of these data is taken from the paper of Hier *et al.* (7).

† Sample taken from middle of horn. A sample from the horn tip assayed 15.7 per cent cystine.

Achilles tendon is characterized by its high content of collagen from which gelatin is derived. For comparison, the amino acid content of gelatin is given in Column 2. There are no great differences in the amino acid content of these two materials.

The principal constituent of yellow tendon is elastin and these two preparations are compared in Columns 3 and 4. It will be noted that both are high in valine in contrast to Achilles tendon and gelatin. Yellow tendon also differs from Achilles tendon in that it contains less arginine, aspartic acid, and lysine but more isoleucine, leucine, phenylalanine, and valine.

Our results for the leucine-isoleucine content of elastin are much lower than those reported in the literature. Thus, Abderhalden and Schitten-

helm (13) report a leucine content of 21.38 per cent, and Hawk *et al.* (4) indicate a combined leucine-isoleucine value of 31 per cent. Block and Bolling (1) report a value of 28 per cent for the total of these two amino acids in elastin. Different samples prepared in our laboratory indicate a combined leucine-isoleucine content of about 11 per cent on repeated assays.

Our values for wool compare very well with those recently reported by Lindley (5), except for proline. Our value for this amino acid is higher and compares better with 9.8 per cent and 9.3 per cent reported by Schmidt (3) and Block and Bolling (1) respectively. The sum of our values of 4.5 per cent for isoleucine and 8.1 per cent for leucine compares well with the total for these two amino acids as reported in several sources (1-3). In the case of valine, we find 5.7 per cent and thus agree with the summary tables of Block and Bolling (1), in contrast to the value of 2.8 per cent reported by Abderhalden and Voitinovici (14).

Incomplete data for the amino acid content of feathers are to be found in the literature. Our figures agree with those available (1) and in Table I a more complete assay of this scleroprotein tissue is presented.

For hair, we agree in general with the values of Block and Bolling (1), except for aspartic acid which we find higher. Our values for arginine, glutamic acid, proline, tyrosine, and valine are higher than those recently reported by Beveridge and Lucas (15) for human hair by isolation methods.

Our values for horn agree with those of Block and Bolling (1), except for leucine which we find lower and for aspartic acid which we find higher. Values for methionine and proline have been added.

#### DISCUSSION

According to the literature, the organic matter of Achilles tendon consists mainly of collagen, while that of yellow tendon is principally elastin but contains a small amount of collagen (4). Our analytical figures bear this out.

In the preparation of elastin, collagen is removed as gelatin. Collagen contains a much higher percentage of arginine, aspartic acid, glutamic acid, histidine, lysine, and methionine than yellow tendon. Elastin is relatively lower than yellow tendon in its content of these amino acids, since the collagen has been removed during its preparation. Collagen contains a relatively low percentage of the remaining amino acids and their concentration in elastin is thus similar to that in yellow tendon.

It is of interest that the amino acid patterns of the keratins from mammals, namely sheep wool, hog hair, and cattle horn, resemble one another closely. However, the avian keratin, chicken feathers, differs from these in being lower in arginine, cystine, glutamic acid, histidine, lysine, and tyrosine and slightly higher in isoleucine and valine.

## SUMMARY

Achilles tendon, yellow tendon, elastin, wool, feathers, hog hair, and cattle horn have been analyzed for fourteen amino acids, and the values are compared with those previously reported. In the case of feathers and horn, several values have been added which were not formerly available.

Differences in the amino acid content of Achilles tendon and yellow tendon are noted.

Elastin has been prepared from yellow tendon and the leucine-isoleucine content has been found to be much lower than that reported in the literature.

The authors wish to express their appreciation to Dr. David Klein for his interest and support and to Edward P. Smith, Theodora Jaksibaga, and LaVerne Naugzemis for technical assistance.

*Addendum*—Since this paper was submitted, we have discovered the paper of Stein and Miller (16) on the composition of elastin. Their values for the amino acid content of this protein agree very well with ours except in the case of the "leucine fraction," which they report as 30 per cent. These authors believed that this fraction contained other amino acids in addition to leucine and isoleucine.

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# MEDIA FOR LEUCONOSTOC MESENTEROIDES P-60 AND LEUCONOSTOC CITROVORUM 8081\*

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(Received for publication, August 30, 1948)

In the routine use of typical media for the microbiological determination of the sixteen amino acids standard curves have accumulated which reveal the amounts of each amino acid required for the growth of the assay organism when all other amino acids are present in excess. Thus it was observed that the relative amounts of the various amino acids needed by *Leuconostoc mesenteroides* P-60 did not coincide either with the proportions found in casein, on which many media are based, or with those of any other medium currently used for the determination of amino acids with this organism.

Media containing border line amounts of one amino acid in the presence of a great excess of a related acid are often unsatisfactory for assay purposes; typical imbalances have been observed between serine and threonine (1), aspartic and glutamic acids (2), norleucine and methionine (3), valine and leucine (4), phenylalanine and tyrosine (4), and glycine and alanine (5). The degree of imbalance (excess concentration of one member of the pair) must usually be great before adverse effects upon sensitive organisms become evident, and while gross imbalances seldom appear in the media used for assay purposes, the possibility remained that the cumulative effect of minor imbalances in an ordinary medium might be responsible for some of the irregular responses observed in connection with the determination of amino acids.

The present study deals with the responses of the two strains *Leuconostoc mesenteroides* P-60 and *Leuconostoc citrovorum* 8081 to a medium based on the requirements of the former organism, and with certain modifications in technique which improve the determination of several of the eighteen amino acids that can now be made with these organisms.

## Methods

The basal medium used in all determinations, unless specifically stated otherwise, was Medium VI, Table I. Dry mixtures of the amino acids,

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation and the American Cancer Society.

TABLE I

*Composition of Medium VI and of Certain Other Typical Media Used in Microbiological Determination of Amino Acids\**

Amino acid	Medium VI (7)	Medium III (6)	Dunn <i>et al.</i> (12)	Stokes <i>et al.</i> (13)	Henderson and Snell (11)
	mg.	mg.	mg.	mg.	mg.
DL- $\alpha$ -Alanine.....	200	200	2000	200	1000
L-Arginine·HCl.....	242	100	80	200	200
L-Asparagine.....	400	200	400		
L-Aspartic acid.....	100			100†	500†
L-Cysteine.....	50				
L-Cystine.....		200	120	200	100
L-Glutamic acid.....	300	400	150	100†	1000
Glycine.....	100	100	100		100
L-Histidine·HCl.....	62	100	20	200	100
DL-Isoleucine.....	250	200	150	200	200
DL-Leucine.....	250	200	150	200	200
L-Lysine·HCl.....	250	200	80	100	200
DL-Methionine.....	100	200	40	200	200
DL-Phenylalanine.....	100	100	60	200	200
L-Proline.....	100	50	25	200	100
DL-Serine.....	50	200	80	200	200
DL-Threonine.....	200	200	450	200	200
DL-Tryptophan.....	40	100	20‡	400	200
L-Tyrosine.....	100	100	30	200	100
DL-Valine.....	250	200	150	200	200
DL-Norleucine.....			100	200	
L-Hydroxyproline.....			100	200	
DL-Norvaline.....			100		
	gm.	gm.	gm.	gm.	gm.
Total weight.....	3.1	3.05	4.4	3.7	5.0
	gm.	gm.	gm.	gm.	gm.
Glucose.....	25	20	20	10	20
Sodium acetate.....	20	20	12	6	1
“ citrate.....					20
Ammonium chloride.....	3		6		3
	mg.	mg.	mg.	mg.	mg.
KH <sub>2</sub> PO <sub>4</sub> .....	600	500	500	500	
K <sub>2</sub> HPO <sub>4</sub> .....	600	500	500	500	5000
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	200	200	200	200	800
FeSO <sub>4</sub> ·7H <sub>2</sub> O.....	10	10	10	10	40
MnSO <sub>4</sub> ·4H <sub>2</sub> O.....	20	10	10	10	160
NaCl.....	10	10	10	10	40
Adenine sulfate·H <sub>2</sub> O.....	10	10	12	10	10
Guanine·HCl·2H <sub>2</sub> O.....	10	10	12	10	10

TABLE I—*Concluded*

Amino acid	Medium VI (7)	Medium III (6)	Dunn <i>et al.</i> (12)	Stokes <i>et al.</i> (13)	Henderson and Snell (11)
	mg.	mg.	mg.	mg.	mg.
Uracil.....	10	10	12	10	10
Xanthine.....	10	10			10
Thiamine·HCl.....	0.5	0.5	1	0.2	1
Pyridoxine·HCl.....	1.0	1.0	1.6		
Pyridoxamine·HCl.....	0.3			0.4	
Pyridoxal·HCl.....	0.3				0.2
Ca <i>dl</i> -pantothenate.....	0.5	0.5	2	0.2	1.0
Riboflavin.....	0.5	0.5	2	0.2	1.0
Nicotinic acid.....	1.0	1.0	2	0.2	1.0
<i>p</i> -Aminobenzoic acid.....	0.1	0.1	0.001	0.04	0.2
Biotin.....	0.001	0.001	0.005	0.0002	0.01
Folic acid.....	0.01	0.01	0.002	0.002	0.01
	ml.	ml.	ml.	ml.	ml.
Distilled H <sub>2</sub> O to.....	500	500	500	500	500

\* In any assay 1 ml. of the basal media described is diluted with 1 ml. of water<sup>†</sup> or of sample.

† Used DL form at 2 times the concentration stated.

‡ Used L form at half the concentration stated.

dextrose, purines, and pyrimidines were ground in a mortar and stored in the refrigerator in tightly stoppered bottles. This has proved to be more economical than the preparation of a series of solutions, since the latter tend to deteriorate on standing. All amino acids used as standards were dried for 2 hours at 60° under 20 mm. of Hg.

*Leuconostoc mesenteroides* P-60 and *Leuconostoc citrovorum* 8081 were maintained in stab culture on a medium composed of 1.5 per cent agar, 1 per cent glucose, and 1 per cent Difco Bacto-yeast extract, and transfers to fresh medium were made at least once a month. Prior to use in assays, the microorganisms were transferred to broth consisting of Medium IV (6), plus 0.2 per cent of yeast extract.

In all assays based on acid production, the final volume used was 2 ml. The tubes were inoculated (7), placed in the incubator at 37° for 72 hours, and the production of acid determined by electrometric titration with 0.02 *N* NaOH. For turbidimetric measurements a final volume of 10 ml. was used, the tubes were incubated for 20 hours at 37°, and cell growth was determined with the 660  $\mu$  filter of the Evelyn colorimeter (6, 8).

*Analysis of Casein*—Casein was dried in a weighing bottle for 3 hours at 60° under 20 mm. of Hg. Approximately 200 mg. of the dried sample were placed in a 25 × 200 mm. Pyrex test-tube and 10 ml. of 4 *N* HCl

added. The tube was then sealed and autoclaved for 8 hours under 15 pounds pressure. For alkaline hydrolysis 10 ml. of 4 N NaOH were used, and the time of autoclaving was increased to 15 hours. Recovery samples consisted of weighed amounts of eighteen amino acids added to samples of casein, which were then autoclaved with either acid or alkali. After hydrolysis all samples were neutralized to a pH of 6.8, and diluted to the desired volume (6). The percentage of nitrogen in casein was determined by the boric acid modification of the macro-Kjeldahl procedure (9); the percentage of ash, by ignition at 1000° in a muffle furnace.

*Composition of Medium VI (Table I) and Comparison with Certain Other Media*—The levels of most of the amino acids in Medium VI represent 10 times the highest level used to promote maximal growth, as indicated in previous standard curves. The level of alanine in the medium is 10 times that needed in the absence of pyridoxamine or pyridoxal, even though these forms of vitamin B<sub>6</sub> are present in Medium VI; alanine does not appear to be required by *Leuconostoc mesenteroides* when pyridoxamine and pyridoxal are in the medium (10). The amount of DL-serine in Medium VI, 100  $\gamma$  per 2 ml. tube, is only 2.5 times the highest level used in the standard curve in the absence of pyridoxal and pyridoxamine. This concentration of serine does not interfere with the assay for threonine (see below), and it is more than adequate for the needs of the organism, since in the presence of pyridoxamine and pyridoxal the requirement of *L. mesenteroides* for serine is very low. The amount of aspartic acid in Medium VI is only 5 times the observed maximum in assay curves but the asparagine present also has some aspartic acid activity for *L. mesenteroides*, and an excess of aspartic acid may interfere with the determination of glutamic acid (2).

Medium VI contains most of the substances found in the other media (Table I) and the total amounts of the amino acids present are also fairly similar. In terms of the isomers used in Medium VI, the total amounts of amino acids in 500 ml. of medium range from 5.0 gm. of amino acid in the medium of Henderson and Snell (11), which was selected on the basis of acid production by *Streptococcus faecalis*, to 3.1 gm. per 500 ml. in Media III and VI. When the concentrations of all the amino acids in this latter medium were doubled, unfavorable effects were noted with *L. mesenteroides* on the standard curves for glutamic acid and lysine. The relative proportions of certain amino acids vary greatly among the five media, the most conspicuous variations being the high amounts of alanine, 46 and 20 per cent in the media of Dunn *et al.* (12) and Henderson and Snell (11), respectively, and the relatively high amounts of aspartic acid in the media of Stokes *et al.* (13) and of Henderson and Snell (Table I).

The new Medium VI differs from Medium III in that it contains aspartic

acid (except when glutamic acid is being determined) and in that the amounts of serine and tryptophan are lowered to 25 and 40 per cent of their former levels, respectively. Cysteine replaces cystine because of its greater solubility. The amount added is also lower in Medium VI than in Medium III, in line with the observed requirement of *Leuconostoc mesenteroides* for this amino acid. The amount of asparagine in Medium VI is double that of Medium III. Asparagine appears to play a specific desirable rôle in bacterial metabolism (14), and irregularities in the standard curves for lysine and glutamic acid (6) have been observed on media containing insufficient amounts of asparagine.

*Acid Production Obtained on Medium VI*—The total amount of acid produced by *Leuconostoc mesenteroides* P-60 in 2 ml. of the complete Medium VI was 17 ml. of 0.02 N acid (Table II), at least as much as that reported on any other medium (15). In the assay of equivalent amounts of certain amino acids, *e.g.* serine and aspartic acid, the production of acid on Medium VI was double that reported by others (16, 17). Maximal acid production corresponding to the usable portions of the various standard curves ranged from 11 ml. for threonine to 15.3 ml. for serine, with a mean of 13.1 ml. of 0.02 N acid for the seventeen amino acids determined. The net acid production (gross titration minus blank) corresponding to the usable portions of the standard curves ranged from 9.5 ml. (glycine assay) to 14.1 ml. of 0.02 N acid (serine assay) (Table II).

The concentrations of the various amino acids necessary for half maximal acid production,  $(17 \text{ ml.} - \text{blank})/2 + \text{blank}$ , are indicated in Column 5, Table II. These concentrations indicate the approximate sensitivities to be expected in the determination of the various amino acids with *Leuconostoc mesenteroides* on Medium VI. The organism was most sensitive to tryptophan, as only 2.6  $\gamma$  per 2 ml. were required for half maximal titration. Other amino acids required in small amounts were phenylalanine, cystine, and histidine, of which 4  $\gamma$  or less per 2 ml. were needed for half maximal acid production. The amino acids required in the largest amounts, 22 to 30  $\gamma$  per 2 ml., were arginine, glutamic acid, and lysine. All other amino acids yielded comparable responses at intermediate concentrations.

Of the various DL- and D-amino acids tested, only D-aspartic acid appeared to be fully as active as the L form for *Leuconostoc mesenteroides* in the lower portion of the curve (Table II); in the upper part of the curve DL-aspartic acid was somewhat less effective in stimulating acid production than the L form. This agrees with the observations of Stokes and Gunness (18) for *Lactobacillus delbrueckii*. A partial activity of D isomers was observed for methionine, serine, and tyrosine, the estimated activities of the D isomers being 10, 12, and 19 per cent of the activities of the corresponding L forms. Other investigators (19, 20) have reported complete inactivity

of D-methionine for *L. mesenteroides*. The activity observed in the present study did not appear to be due to the presence of L-methionine in the sample of the D isomer tested, as the optical rotation<sup>1</sup> was  $[\alpha]_D^{23} = -25.40^\circ$  ( $[\alpha]_D^{25} = -21.18^\circ$ ) (21), and the sample had no activity for *L. citrovorum*. The observed activity of the D-serine, however, could have been due to con-

TABLE II

*Acid Production by Leuconostoc mesenteroides P-60 on Medium VI Containing Graded Amounts of Each of Seventeen Amino Acids*

Amino acid	Standard curve* $\gamma$ per 2 ml.	0.02 N acid produced per 2 ml.			Amino acid per 2 ml. for half maximal titration $\gamma$	Activity of	
		Blank	Gross	Net		DL form	D form
		ml.	ml.	ml.		per cent	per cent
Arginine.....	0-40	2.0	11.8	9.8	30		
Aspartic acid.....	0-40	1.8	14.3	12.5	20	100	
Cystine.....	0-10	1.4	14.2	12.8	3	<50†	
Glutamic acid.....	0-60	1.2	12.5	11.3	30	50	
Glycine.....	0-25	4.2	13.7	9.5	10		
Histidine.....	0-10	1.4	14.9	13.5	4	50	
Isoleucine.....	0-25	1.3	12.6	11.3	14	50	0.0‡
Leucine.....	0-25	1.2	12.5	11.3	13	50	
Lysine.....	0-40	1.8	13.1	11.3	22	50	
Methionine.....	0-10	1.4	12.0	10.6	6	54	<10‡
Phenylalanine.....	0-10	2.5	15.0	12.5	3	50	0.0‡
Proline.....	0-20	1.0	14.5	13.5	4.5	46	
Serine.....	0-20	1.2	15.3	14.1	8	50	12‡
Threonine.....	0-20	0.9	11.0	10.1	14		0.0§
Tryptophan.....	0-4	1.4	11.5	10.1	2.6	49	
Tyrosine.....	0-20	1.7	13.9	12.2	8		19‡
Valine.....	0-25	1.1	12.6	11.5	15.6	50	0.0‡
None omitted.....				17.0			

\* L isomers were used as standards, except for threonine; only the DL form was available.

† Obtained from W. H. Riesen of the Biochemistry Department.

‡ Obtained through the courtesy of Dr. D. Doherty of the Biochemistry Department. We are also indebted to Dr. Doherty for the L-isoleucine, L-valine, and L-serine standards.

§ Obtained from R. J. Sirny of the Biochemistry Department.

tamination, since a sample of DL-serine possessed only 50 per cent of the activity of the L form. Most other amino acids appeared to be active only in the L form, while DL-cystine at high concentrations was somewhat less active than equivalent amounts of the pure L isomer (22).

Data on acid production by *Leuconostoc citrovorum* 8081 on Medium VI are presented in Table III. Since *L. citrovorum* requires a growth factor

<sup>1</sup> Courtesy of Dr. D. Doherty of the Biochemistry Department.

in addition to the known vitamins and amino acids (10), 0.4 ml. of Lilly's liver concentrate (reticulogen, 20 U. S. P.) was added per 500 ml. of medium. With this addition, *L. citrovorum* proved to be satisfactory for the assay of twelve amino acids. This organism responded only to the L isomer of most amino acids, including methionine, and thus was more specific for the latter acid than was *L. mesenteroides*; *L. citrovorum* responded equally well to D- and to L-alanine. *L. citrovorum* proved satis-

TABLE III

*Response of Leuconostoc citrovorum to Twelve Amino Acids and Their Isomers*

Amino acid	Standard curve*	0.02 N acid produced per 2 ml.			Amino acid per 2 ml. for half maximal titration	Activity of	
		Blank	Gross	Net		DL form	D form
	$\gamma$ per 2 ml.	ml.	ml.	ml.	$\gamma$	per cent	per cent
$\alpha$ -Alanine.....	0-50	1.6	14.7	13.1	20.0	100	100†
Arginine.....	0-40	2.3	18.5	16.2	11.6		
Cystine.....	0-5	1.8	15.7	13.9	2.3	<50‡	
Glutamic acid.....	0-60	1.8	13.5	11.7	27.0	50	
Glycine.....	0-25	3.6	13.6	10.0	13.0		
Histidine.....	0-10	2.2	17.0	14.8	4.0	50	
Isoleucine.....	0-25	5.2	17.2	12.0	13.3	50	0.0‡
Methionine.....	0-10	3.0	15.3	12.3	4.6	50	0.0‡
Proline.....	0-20	3.3	18.2	14.9	4.7	47	
Threonine.....	0-20	1.5	15.0	13.5	10.0		0.0§
Tyrosine.....	0-20	2.6	18.5	15.9	6.0		29.0†
Valine.....	0-25	1.9	14.3	12.4	13.0	50	0.0†
None omitted.....				19.0			

\* L isomers were used as standards, except for threonine; only the DL form was available.

† Obtained through the courtesy of Dr. D. Doherty of the Biochemistry Department. We are also indebted to Dr. Doherty for the L-alanine, L-isoleucine, and L-valine standards.

‡ Obtained from W. H. Riesen of the Biochemistry Department.

§ Obtained from R. J. Sirny of the Biochemistry Department.

factory for the assay for proline, while the only organism previously used for this determination was *L. mesenteroides*. *L. citrovorum* appears preferable to other organisms in the assay of arginine because of its relatively low requirement for this amino acid; a half maximal production of acid by *L. citrovorum* was observed in the presence of only 11.6  $\gamma$  of arginine per 2 ml. tube (Table III), as compared with 30  $\gamma$  for *L. mesenteroides* (Table II). *L. citrovorum* was also slightly more sensitive to smaller amounts of glutamic acid than *L. mesenteroides*, and an additional advantage in favor of *L. citrovorum* is its decreased sensitivity to inhibition by aspartic acid

(see below). *L. citrovorum* is particularly valuable in the assay for alanine (10).

### *Special Procedures for Certain Amino Acids*

**Glutamic Acid**—One difficulty encountered in the determination of glutamic acid has been a relatively low production of acid at the lower concentrations of glutamic acid (2, 11, 23). This lag in the standard curve persisted when *Leuconostoc mesenteroides* was grown on Medium VI in which the concentration of asparagine was varied, or in which 2 mg. of pyridoxamine were added per 500 ml. When aspartic acid was omitted from the medium, as suggested by Baumgarten *et al.* (2), satisfactory curves were obtained with *L. mesenteroides*, *Streptococcus faecalis*, *L. citrovorum*, and *Lactobacillus lycopersici*. The addition of moderate amounts of aspartic acid to the medium caused less depression in the growth curve of *L. citrovorum* than in that of *L. mesenteroides*, and as long as the concentration of aspartic acid in the sample did not exceed 3 times that of glutamic acid no inhibition with *L. citrovorum* was noted. Lyman *et al.* (24) have shown that the addition of glutamine to media containing aspartic acid improved the appearance of the standard curve for glutamic acid with, however, a somewhat higher blank. This has been confirmed with *L. mesenteroides* by the addition of glutamic acid itself. This procedure, however, was unnecessary for *L. citrovorum* grown on Medium VI from which aspartic acid has been omitted. Analysis of casein with the latter medium yielded a value of 23 per cent of glutamic acid in agreement with accepted literature values.

**Serine and Threonine**—*Leuconostoc mesenteroides* has been preferred to other organisms for the assay of serine because of its relatively high sensitivity and because the presence of pyridoxamine or pyridoxal in a serine-free medium stimulated the growth of *L. mesenteroides* much less than that of *Streptococcus faecalis*. Another difficulty in the microbiological determination of serine is that high amounts of threonine may depress the response of the organism to serine (16). The amounts of threonine ordinarily encountered in samples, however, are below the critical level. In the presence of 12  $\gamma$  of L-serine per 2 ml. no inhibition resulted when 720  $\gamma$  of DL-threonine were present, while 880  $\gamma$  of DL-threonine resulted in an inhibition of only 5 per cent. Although serine has been determined successfully with *L. mesenteroides* on Medium III (6), the sensitivity of the determination has now been increased somewhat by use of Medium VI, from which pyridoxal and pyridoxamine have been omitted. These forms of vitamin B<sub>6</sub> may be destroyed in a sample by exposure to ultraviolet light for a period of 20 to 30 minutes without detectable destruction of serine.

For the assay of threonine, *Streptococcus faecalis* has been preferred to



other organisms because of its low susceptibility to inhibition by serine (1, 16). In the present study no inhibition of *Streptococcus faecalis* resulted when 480  $\gamma$  of DL-serine were added to 2 ml. of medium containing 12  $\gamma$  of threonine plus 100  $\gamma$  of DL-serine (Table IV), although a 30 per cent inhibition in the growth of *Leuconostoc mesenteroides* resulted when 450  $\gamma$  (350  $\gamma$  plus 100  $\gamma$  in the medium) of DL-serine were present per 2 ml., while a 27 per cent inhibition in the growth of *Leuconostoc citrovorum* was caused by 580  $\gamma$  (480  $\gamma$  added plus 100  $\gamma$  in the medium) of DL-serine in 2 ml. of medium containing 12  $\gamma$  of threonine (Table IV). Only the L form of serine interfered with the assay of threonine.

TABLE IV  
Inhibition of Threonine Utilization by Serine

Organism	DL-Threo- nine per 2 ml. tube	Additional serine* per 2 ml. tube	Per cent inhibition		
			DL-Serine	L-Serine†	D-Serine†
<i>L. mesenteroides</i> P-60.....	$\gamma$	$\gamma$			
	12	50	0	0	0
	12	100		12	0
	12	160	20		
	12	350	30	45	0
<i>L. citrovorum</i> 8081.....	12	560	54		
	12	100	0	15	0
	12	160	2		
	12	300	18	30	0
	12	480	27		
<i>S. faecalis</i> R.....	12	350		0	0
	12	480	0		

\* Medium VI used in all these determinations contains 100  $\gamma$  of DL-serine per ml. (Table I), or 100  $\gamma$  per 2 ml. of diluted medium, as used in the assays.

† We are indebted to Dr. D. Doherty of the Biochemistry Department for these preparations.

Thus *Streptococcus faecalis* remains the organism of choice in the determination of threonine and, while it is possible to use Medium VI for this organism, with suitable changes in the buffer, acid production and sensitivity were found to be greatest when this organism was grown on Medium II (6).

*Glycine*—Both *Leuconostoc mesenteroides* and *L. citrovorum* have been used with fair success for the determination of glycine with Medium VI, although high blank titrations were encountered, 6 to 8 ml. of 0.02 N acid per 2 ml. Analyses of the separate amino acids failed to indicate any gross contamination of the medium with glycine, while the blanks were low in turbidimetric assays. Variations in the concentration of pyrid-

oxamine, pyridoxal, or alanine in Medium VI did not materially alter the blank value. A glycine-alanine imbalance, therefore, did not seem to be involved, although on a medium in which 46 per cent of the amino acids was alanine Shankman *et al.* (4) observed low blanks and a lag in the standard curve which was overcome by the addition of a small amount of glycine to the medium. The nature of the irregularity in the responses of *L.*

TABLE V  
*Amino Acid Content of Casein*

Amino acid	Organism used				Literature values
	<i>L. mesenteroides</i>		<i>L. citrovorum</i>		
	<i>per cent</i> *	<i>per cent recovered</i>	<i>per cent</i> *	<i>per cent recovered</i>	<i>per cent</i>
Alanine.....			3.70	105.0	2.0-5.7 (26)*
Arginine.....	4.14	99.5	3.68	96.6	4.2 (27)*
					3.8 (28)
Aspartic acid.....	7.77	100.5			6.1 (18)
					7.2 (14)
Cystine.....	0.51	103.0	0.57	103.0	0.3 (27)
Glutamic acid.....	23.3	98.5	22.8	98.7	23.2 (2)
Glycine.....	2.21	100.0	2.1	107.0	2.1 (27)
					1.9 (4)
Histidine.....	3.37	105.5	3.34	104.0	3.0 (27)
Isoleucine.....	6.60	101.0	6.60	92.0	6.5 (27)
Leucine.....	10.50	104.0			9.9 (27)
Lysine.....	8.6	107.0			7.9 (27)
Methionine.....	3.3	100.0	2.9	105.0	3.5 (27)
Phenylalanine.....	5.4	98.0	5.3	96.0	5.6 (27)
Proline.....	11.7	100.5	12.2	110.0	11.6 (11)
Serine.....	6.35	102.0			6.4 (18)
Threonine†.....	4.77	95.0	4.60	105.0	4.1 (27)
Tryptophan.....	1.39	109.0			1.2 (27)
Tyrosine.....	6.30	152‡	5.9	154‡	6.5 (27)
Valine.....	7.60	100	7.80	103	6.7 (27)

\* Calculated to 16 gm. of N<sub>2</sub>, ash-free, moisture-free.

† *Streptococcus faecalis* value, 4.55 per cent in casein; recovery 100 per cent.

‡ See the text.

*mesenteroides* and *L. citrovorum* to glycine in Medium VI remains to be clarified, but tentative values for glycine can be obtained after an incubation period of 48 hours instead of the customary 72 hours.

*Application of New Media to Analysis of Casein*—The percentage composition of casein for eighteen amino acids was determined with two organisms, *Leuconostoc mesenteroides* and *Leuconostoc citrovorum* on Medium VI or on the appropriate variants of this medium. The percentage

recoveries were obtained by adding a mixture of eighteen amino acids in amounts supplying approximately as much of the amino acid in question as in the sample. Table V indicates that the values were all within or close to the accepted range, and that recoveries were generally satisfactory. Tyrosine, added to a medium in the free form, is racemized much more slowly under ordinary hydrolysis treatment than tyrosine released from protein (25) and hence the apparent recoveries of this acid are high.

#### SUMMARY

1. A new medium for the microbiological assay of amino acids was based upon the relative amounts of the amino acids required for the growth of *Leuconostoc mesenteroides* P-60. The medium was used for the determination of eighteen amino acids with either *L. mesenteroides* or *L. citrovorum* 8081. Acid production on the medium for all amino acids was at least as high as those previously recorded for *L. mesenteroides*, while for aspartic acid and serine acid production exceeded reported values.

2. The D forms of most amino acids proved to be inactive for both *Leuconostoc mesenteroides* and *L. citrovorum*. DL-Aspartic acid was equal in activity to L-aspartic acid for *L. mesenteroides*, while D-methionine, D-serine, and D-tyrosine had partial activity. Both D- and DL-alanine were as active as L-alanine for *L. citrovorum*; D-tyrosine was only partially active.

3. Details are presented for the improved determination of serine, threonine, glycine, and glutamic acid. The methods are applied in the analysis of casein for eighteen amino acids.

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## A MICROBIOLOGICAL DETERMINATION OF ALANINE\*

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(Received for publication, August 30, 1948)

In the past alanine has been determined mainly by isolation or by chemical techniques (1). Such procedures are usually tedious, of low sensitivity, and often of doubtful specificity. Only two instances have been recorded of the microbiological determination of alanine (2, 3); *Streptococcus faecalis* was used as the assay organism, but the experimental details were not revealed. The present report deals with the microbiological determination of alanine with *Leuconostoc citrovorum* 8081. Numerous other organisms were investigated as to their suitability for the assay of alanine, including *Lactobacillus casei*, *L. delbrueckii* 3, *L. brevis*, *L. fermentii*, *L. pentosus*, *L. arabinosus*, *L. lycopersici*, *L. brassicae*, *S. faecalis*, and *Leuconostoc mesenteroides* P-60. Most of these organisms produced nearly as much acid on media devoid of alanine as on those containing it. Impaired growth in the absence of alanine, when it was observed at all, was usually found only during the initial phases of the growth period. The requirement for alanine by *S. faecalis* and *L. mesenteroides* P-60 was nearly eliminated with the amounts of pyridoxal and pyridoxamine present in Medium VI (4), which is useful in the determination of fifteen other amino acids. Even in the absence of pyridoxal and pyridoxamine, the blank titrations with *L. mesenteroides* were so high that an assay for alanine was impractical, while with *S. faecalis* the titrations were too variable for use in a standard curve. Furthermore, *S. faecalis* responded irregularly to D-alanine and the response to low amounts of alanine could be inhibited by glycine (5).

*Lactobacillus lycopersici* was found to be a somewhat better organism for the assay of alanine, although far from ideal. The response of this organism to alanine was determined turbidimetrically with the 660 m $\mu$  filter of the Evelyn colorimeter, since acid production by the organism was poor. Variations in response were great and a long period of incubation was required unless the inoculum used was heavy and taken from a vigorously growing culture of the organism. Moreover, pyridoxamine and pyridoxal stimulated growth in the absence of alanine, and with sufficient

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation and the American Cancer Society.

time the organism grew well, even in the absence of these forms of vitamin B<sub>6</sub>. The organism ferments xylose more readily than glucose and hence for reliable results samples to be assayed must be free of xylose. The use of xylose as the fermentable carbohydrate presents other disadvantages, such as caramelization during autoclaving. Nevertheless, the values obtained with *L. lycopersici* for the alanine content of casein (3.7 to 4.1 per cent, average 3.9 per cent, corrected to 16 per cent nitrogen, ash- and moisture-free protein) compared favorably with the values obtained later with *L. citrovorum* (see below).

*Response of Leuconostoc citrovorum to Alanine*—*Leuconostoc citrovorum* 8081 failed to grow on synthetic Medium VI (4) unless an unidentified growth factor was present (6). This factor could be furnished in sufficient

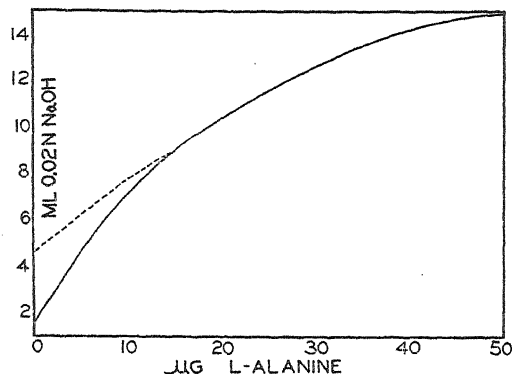


FIG. 1. Response of *Leuconostoc citrovorum* to graded amounts of L-alanine. Broken line, medium containing pyridoxal or pyridoxamine; solid line, medium containing no pyridoxal or pyridoxamine. Incubation time, 72 hours; volume, 2 ml.

amount by adding 0.4 ml. of a commercial liver preparation (Lilly's reticulo-gen, 1 U. S. P. unit per 0.05 ml.) to 500 ml. of the synthetic Medium VI. This amount permitted optimal growth of the organism (6) when 50  $\gamma$  of alanine were present per tube (1 ml. of basal medium, plus 1 ml. of water or sample per tube), whereas little growth was observed when alanine was omitted from the medium containing the liver concentrate. By the addition of the liver concentrate to Medium VI, plus graded amounts of alanine, a satisfactory standard curve was obtained (Fig. 1). The response to the added amounts of alanine could be measured satisfactorily either turbidimetrically after 15 to 18 hours of incubation at 37° or by electrometric titration of the acid produced after 72 hours. The usable portion of the standard curve ranged from 0 to 25  $\gamma$  of L-alanine per ml. The effects of pyridoxamine and pyridoxal on the determination were found to be less serious with *Leuconostoc citrovorum* than with the other organisms studied.

However, the amounts of acid produced on alanine-free media containing pyridoxal or pyridoxamine reached about one-third the maximum possible on media containing alanine (Table I), although increasing amounts of pyridoxal or pyridoxamine failed to increase acid production further. Thus the effect of pyridoxal and pyridoxamine was sufficiently great to warrant caution that samples to be analyzed microbiologically be low in these forms of vitamin B<sub>6</sub>.

Since the sensitivity and range of the standard curve were increased somewhat in the absence of pyridoxal and pyridoxamine, these forms of vitamin B<sub>6</sub> were omitted from the medium in the analysis of samples known to be low in these compounds; *e.g.*, purified proteins. However, when the presence of pyridoxal or pyridoxamine in the sample was suspected, these sub-

TABLE I

*Effect of Pyridoxamine and Pyridoxal upon Acid Production by Leuconostoc citrovorum on Medium Devoid of Alanine*

Pyridoxamine per 2 ml.	0.02 N acid produced	Pyridoxal per 2 ml.	0.02 N acid produced	Alanine per 2 ml.	0.02 N acid produced
$\gamma$	ml.	$\gamma$	ml.	$\gamma$	ml.
0	2.3	0	2.3	0	2.3
0.4	3.7-4.2	0.4	3.9-5.8	5	4.9
0.8	4.3-4.9	0.8	5.3-5.9	10	7.3
1.2	5.5	1.2	7.7	15	9.2
2.0	5.2-5.4	2.0	4.8-8.7	20	10.7
4.0	5.3-6.3	4.0	7.2-9.3	30	13.3
6.0	4.9	6.0	5.3	40	15.1
100	7.4	100	4.7	50	16.2

stances were destroyed by exposure of the samples to sunlight or to ultraviolet light for a period of 20 to 30 minutes. This procedure has also been used successfully in the assay of serine.

The comparative activities of DL-alanine, D-alanine, and L-alanine were determined at graded concentrations of 0 to 25  $\gamma$  per ml. of the various isomers. The purity of the D-alanine<sup>1</sup> and L-alanine<sup>1</sup> was determined by optical rotation, and a sample of commercial DL-alanine was recrystallized twice from hot water. These samples were then dried to constant weight at 55-60° and 20 mm. of Hg. Both DL-alanine and D-alanine were found to be approximately as active for *Leuconostoc citrovorum* as L-alanine (Table II); the presence or absence of pyridoxal and pyridoxamine in the medium did not affect the relative activities of the isomers, while sodium pyruvate and  $\beta$ -alanine were inactive.

<sup>1</sup> The samples of D-alanine,  $[\alpha]_D^{25} = -15.4^\circ$ , and L-alanine,  $[\alpha]_D^{25} = +15.3^\circ$ , used in this study were obtained from Dr. M. Stahmann of this department.

*Alanine Content of Certain Proteins*—Hydrolysates of nine representative proteins were prepared by placing approximately 200 mg. of the vacuum-dried protein in a Pyrex test-tube, adding 10 ml. of 4 N HCl, and sealing and autoclaving the contents at 15 pounds pressure (121°) for 8 hours. Recovery samples were prepared by adding known amounts of a mixture of eighteen amino acids to samples of casein and hydrolyzing as above. Alkaline hydrolysates and recovery samples were also prepared by substituting 10 ml. of 4 N NaOH for the hydrochloric acid and extending the hydrolysis period to 15 hours.

The alkaline hydrolysis of casein resulted in high values for alanine (5.4 per cent) and also in high recovery values (196 per cent), suggesting that some of the other amino acids may be converted into alanine-like com-

TABLE II  
*Acid Production by Leuconostoc citrovorum in Presence of L-, DL-, and D-Alanine (72 Hours Incubation)*

Alanine per 2 ml.	Acid (0.02 N) produced per 2 ml.		
	L-Alanine	DL-Alanine	D-Alanine
$\gamma$	ml.	ml.	ml.
0	1.6	1.6	1.6
5	4.7	4.3	4.0
10	7.5	7.2	6.2
15	8.8	8.8	8.3
20	10.4	10.3	9.7
25	11.5	11.2	10.8
30	12.6	12.6	12.2
40	14.2	13.8	13.5
50	14.7	14.7	14.6

pounds during alkaline hydrolysis. On the other hand, recoveries of alanine added before hydrolysis with acid ranged from 88 to 115 per cent, with an average of 105 per cent for six separate assays. The alanine content of casein was found to range from 3.3 to 4.2 per cent (ash-free, moisture-free, 16 per cent nitrogen), with an average value of 3.7 per cent (Table III). Samples of casein hydrolyzed with HCl for 12 hours yielded the same value for alanine as samples hydrolyzed for 8 hours. The percentages for alanine in the other proteins ranged from 3.5 per cent, in wheat gluten, to 13.0 per cent in zein. The amounts of alanine found in the various proteins by the new microbiological procedure generally fell within the range found by other investigators who used chemical procedures, although for gelatin and zein the discrepancies between the two types of analysis were great. The results of the chemical analyses, however, are



regarded as "at best approximations to the true quantities" (1). Incidentally, the values obtained for the alanine content of the various proteins were essentially the same, whether the assays were conducted on media containing the three forms of vitamin B<sub>6</sub> or only pyridoxine (Table III). Under routine conditions of analysis, therefore, the presence of pyridoxamine or pyridoxal in the medium does not seriously interfere with the accuracy of the determination.

*Alanine Content of Certain Normal and Neoplastic Tissues*—Representative normal and neoplastic tissues (Table IV) were dried at 55–60° and 20

TABLE III  
*Alanine Content of Various Proteins*

Protein	Nitrogen	Alanine content		
		Medium containing no pyridoxal or pyridoxamine	Medium containing all 3 forms of vitamin B <sub>6</sub>	Literature (1)
	<i>per cent</i>	<i>per cent</i> *	<i>per cent</i> *	<i>per cent</i> *
Casein (HCl-hydrolyzed).....	14.5	3.7	3.7	2-5.7
“ (NaOH-hydrolyzed).....	14.5	6.6	7.6	
Hemoglobin.....	15.5	10.6	9.8	7-8
Wheat gluten.....	13.5	3.5	2.7	5
Zein.....	15.1	13.0	12.3	5-10
Fibrin.....	15.2	4.1	4.0	
Lactalbumin.....	13.3	7.0	6.6	
Edestin.....	16.0	3.9	4.0	3-4.8
Gelatin.....	17.1	8.5	8.0	6-22
Egg albumin.....	13.4	7.9	7.6	7.4

\* Calculated to 16 per cent nitrogen, ash-free, moisture-free protein. We are indebted to Betty F. Steele for these determinations.

mm. of Hg and the fat was extracted with absolute ether for 10 hours in a continuous extraction apparatus. The fat-free residues were hydrolyzed in sealed Pyrex tubes with 4 N HCl for 8 and 12 hours, and the hydrolysates were then neutralized, diluted, filtered, and analyzed for alanine with *Leuconostoc citrovorum*.<sup>2</sup> The percentages of alanine in the normal tissues analyzed ranged from 4.7 per cent in liver to 7.4 per cent in muscle (fat-free, moisture-free tissue, corrected to 16 per cent nitrogen). The percentages of alanine in the various types of tumors analyzed ranged from 6.0 per cent for a subcutaneous rabbit tumor to 8.5 per cent for a spontaneous mammary tumor of a rat. Variations in food intake and the exposure of rats to certain azo dyes appeared to modify the alanine content of the tis-

<sup>2</sup> Mr. C. E. Blades assisted with these determinations.



3. The amounts of alanine found in seven different samples of tumors ranged from 6.0 to 8.5 per cent of the fat-free dry material; in normal tissues the amounts ranged from 4.7 to 7.8 per cent.

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# THE ACTION OF AMINOBENZYLTHIAZOLIUM SALTS ON THIAMINE DESTRUCTION BY THE CHASTEK PARALYSIS ENZYME\*

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(Received for publication, August 31, 1948)

The demonstration that certain thiamine analogues specifically and competitively inhibit the thiamine-destroying enzyme of fish tissues afforded an additional method for characterizing the action of the enzyme and analyzing the structural features of the inhibitor molecules in relation to their effectiveness. *o*-Aminobenzyl-(3)-4-methylthiazolium chloride at  $5 \times 10^{-4}$  M concentration was found to produce 100 per cent inhibition of the enzymatic destruction of  $5 \times 10^{-4}$  M thiamine (1). With the same concentration of the *o*-nitro compound corresponding to this amine no inhibition was encountered. The suggested importance of the amino group was further substantiated by the finding that  $\beta$ -aminoethyl-(3)-4-methylthiazolium chloride at the above concentration caused 56 per cent inhibition, whereas its corresponding phthalimido derivative produced only 18 per cent inhibition. In the same study 6-aminopyrimidine compounds were likewise employed. Since they proved decidedly less effective in inhibitory activity, it was tentatively concluded that components other than the benzylamine moiety of the thiazolium compounds were of importance in the inhibition process. In any case, the application of the Lineweaver and Burk (2) treatment of the Michaelis and Menten (3) concept of enzyme action yielded an enzyme-inhibitor dissociation constant of  $1.97 \times 10^{-6}$  mole per liter for the *o*-aminobenzylthiazolium salt. In the same experiment a Michaelis constant of  $8.31 \times 10^{-5}$  mole per liter was obtained for the enzyme-substrate (thiamine) complex.

With this quantitative evidence of specific and competitive inhibitory activity, this analogue which so closely resembles the structural pattern of the vitamin was selected as the point of demarcation for the more detailed analysis of the essential characteristics of an inhibitor molecule. The task of selecting the points of alteration is greatly simplified with the

\* A preliminary report of a portion of this work was presented before the Division of Biological Chemistry of the American Chemical Society at Cleveland, April 4, 1944.

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realization that this compound contains the benzyl instead of the pyrimidine ring and lacks the 2-methyl group characteristic of the vitamin pyrimidine and the 5- $\beta$ -hydroxyethyl side chain of the thiazole ring. Consequently, the rôle of the amino group, its placement relative to the methylene bridge between the two rings, and the effect of altering the 4-methyl group have been analyzed. The compounds selected for this study included quaternary benzyl, and *o*-, *m*-, and *p*-aminobenzyl derivatives of 2-, or 4-methyl-, or 2,4-dimethylthiazole. Their synthesis has been readily accomplished (4) by the method of Clarke (5) and it is the purpose of this paper to record the results of their use in connection with the thiamine-destroying enzyme.

#### EXPERIMENTAL

The enzyme was prepared for these experiments by extracting acetone-desiccated powder (6) of carp (unless otherwise indicated) viscera and gills with several portions of 10 per cent sodium chloride in 0.2 M phosphate buffer of pH 7.4. After adjustment to pH 7.4 and dilution to the desired volume, 2 ml. aliquots, representing or equivalent to the weight of powder indicated in each case, were used. In actual practice, approximately 50 per cent of the weight of the powder dissolves in the medium. Water and the inhibitor solution were added to make a total volume of 4 ml. 1 ml. of  $25 \times 10^{-4}$  M thiamine (2.5 micromoles) was added and the solutions incubated for 2 hours at 37.5°. The reaction was stopped with the addition of 5 ml. of 10 per cent trichloroacetic acid. The thiamine remaining was determined by means of the Melnick-Field method (7), comparison in each case being made with an identical unincubated solution. The per cent inhibition was calculated in the usual fashion.

Since the *o*-aminobenzylthiazolium chloride had previously been shown to be an effective inhibitor, the first comparison was made with the modifications involving only the alteration of the thiazole ring. In the previous communication (1) the 4-methyl derivative was employed. In Table I are presented illustrative results obtained with the 2-methyl and the 2,4-dimethyl compounds as well as the original compound. In this comparison, it is obvious that the 4-methyl group is not a critical feature of the inhibitor molecule, for with it in the 2 position an equally active or slightly better inhibitor is obtained. However, with a methyl radical in both the 2 and 4 positions of the thiazole ring, a very marked difference in inhibitory action is observed. From these results it may be inferred that neither position is of real importance in the combination of enzyme and inhibitor molecules. Possibly the decreased effectiveness of the dimethyl derivative is merely an instance of steric hindrance with subsequent decreased combination with the enzyme.

Of more importance is the placement of the amino group in the benzyl

portion of the molecule. In the first place, evidence previously cited (1) is entirely substantiated. In the typical experiment presented in Table II,

TABLE I  
*Inhibition with o-Aminobenzylthiazolium Compounds*

Each test-tube contained the equivalent of 400 mg. of mullet tissue, Preparation 7-102A, the incubations being carried out as described in the text.

Thiazole substituent investigated	Concentration	Inhibition
	<i>moles per l. <math>\times 10^4</math></i>	<i>per cent</i>
2-Methyl	2.5	86.1
	5.0	89.2
	10.0	90.6
4-Methyl	2.5	64.4
	5.0	67.5
	10.0	73.7
2,4-Dimethyl	2.5	18.7
	5.0	30.2
	10.0	42.6

TABLE II  
*Effect of m- and p-Aminobenzylthiazolium Salts*

Each test-tube contained the equivalent of 400 mg. of mullet tissue, Preparation 7-102A.

Compound added	Concentration	Thiamine destroyed	Inhibition (−) or activation (+)
	<i>moles per l. <math>\times 10^4</math></i>	<i>micromoles</i>	<i>per cent</i>
None		1.64	
<i>o</i> -Amino-4-methyl	5.0	0.36	−78.1
<i>m</i> -Amino-4-methyl	2.5	1.92	+17.5
	5.0	2.30	+41.0
	10.0	2.45	+50.0
<i>p</i> -Amino-4-methyl	2.5	1.66	+2.0
	5.0	1.61	−1.7
	10.0	1.57	−3.6
<i>m</i> -Amino-2-methyl	2.5	2.17	+33.0
	5.0	2.36	+44.0
	10.0	2.40	+47.0
<i>p</i> -Amino-2-methyl	2.5	1.72	+6.0
	5.0	1.67	+2.0
	10.0	1.60	−2.0

1.64 micromoles of thiamine were destroyed by the enzyme when no added compound was present. With the inclusion of *o*-aminobenzyl-4-methylthiazolium chloride at the same concentration, only 0.36 micromole of

thiamine was destroyed. In contrast, alteration of the inhibitor molecule by removal of the amino group from the ortho to the meta position causes complete loss of inhibitory activity. In place of inhibition, an entirely unexpected finding of activation by *m*-aminobenzyl-4-methylthiazolium chloride was observed. Whereas 1.64 micromoles of thiamine disappeared in the control solution, 1.92, 2.30, and 2.45 micromoles of thiamine disappeared in the presence of increasing quantities of the meta derivative. The increased destruction in the three instances yields a calculated activation of 17.5, 41.0, and 50.0 per cent, respectively. This activation has been observed in numerous other experiments with the same compound and is substantiated by the results obtained with the *m*-aminobenzyl-2-methylthiazolium compound in a large variety of experiments. Illustrative results with this compound are included in Table II. The degree of activation is essentially the same with the two compounds; so that the phenomenon may be regarded as independent of a 2- or 4-methyl group as is inhibition. It should be observed also that in each case increasing concentration results in increasing activation.

The *m*-aminobenzyl-2,4-dimethylthiazolium chloride is like the other meta compounds in that activation results. In one experiment, for example, 48.8 per cent activation was obtained with 2.5 micromoles, whereas the analogous 4-methyl derivative yielded 54.0 per cent activation. In this case at least the presence of the second methyl group did not reduce the effect obtained, as it does in the case of inhibition.

With the *p*-amino derivative, whether it contains the 2-methyl- or the 4-methylthiazole ring, little or no effect on the enzymatic reaction is obtained, as is illustrated in the results presented in Table II. The para compound thus furnishes unqualified evidence that the ortho position of the benzyl nucleus must be substituted with the amino group for effective inhibition of the enzymatic destruction of thiamine.

As still further proof of this point, similar experiments were carried out with benzyl-4-methylthiazolium chloride, in which no amino radical is present. In every instance the same destruction of thiamine occurred in the control and experimental tubes. Significant inhibition or activation was not observed in numerous experiments, two of which are included in Table III.

One point of difference between certain results described here and previously published results has led to important findings with respect to inhibition. In the paper by Sealock and Goodland (1) it was reported that  $5 \times 10^{-4}$  M *o*-aminobenzyl-4-methylthiazolium chloride completely inhibited the enzyme activity of the extract used. In this instance (Table II) the same concentration of this compound yielded only 78 per cent inhibition with a different preparation (No. 7-102A). With still another



preparation (No. 7-113A) 20 per cent inhibition was observed. Differences in per cent inhibition of this order of magnitude are not commonly encountered with the same ratio of inhibitor to substrate concentrations, and indeed are theoretically impossible on the basis of the classical Mi-

TABLE III

*Benzyl-4-Methylthiazolium Chloride and Thiamine Destruction*

Mullet viscera, Preparation 7-102A, was used in an amount equivalent to 100 mg. in Experiment I and to 400 mg. in Experiment II.

Compound added	Thiamine destruction	
	Experiment I	Experiment II
	per cent	per cent
micromoles		
None	20.6	65.3
1.25	22.4	67.2
2.50	22.0	66.3
5.0	23.2	60.2

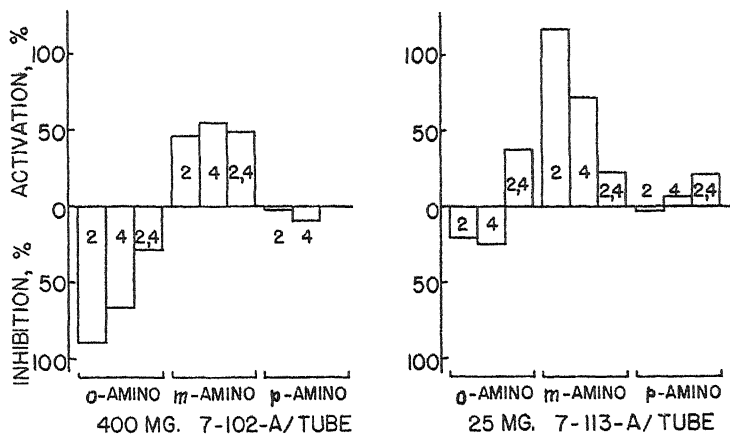


FIG. 1. Effect of benzylthiazolium chlorides on thiamine destruction by two different enzyme preparations. The numbers accompanying the bars indicate the positions of the methyl groups substituent to the thiazole ring. The amine groups are attached to the benzyl ring as indicated at the bottom of each graph. The preparations were obtained from mullet (left) and carp (right), and in each test thiamine and the thiazolium compound were used at  $5 \times 10^{-4}$  M.

chaelis-Menten concept of enzyme reaction. However, these differences did occur and were obtained repeatedly. Likewise, the other inhibitors behaved in the same fashion. It was therefore assumed that some unknown factor was operating.

In a search for this factor, comparative experiments of the type illustrated in Fig. 1 were carried out with two different enzyme preparations.

In each case  $5 \times 10^{-4}$  M concentration of inhibitor or activator compound was employed. The differences in inhibition obtained with the three *o*-amino compounds are readily observed. Likewise, there also appears to be some difference in the activation observed with the *m*-amino salts as one compares the two enzyme preparations. At the same time, the *p*-amino compounds were relatively ineffective with both preparations. It should be noted that Preparation 7-102A was used at a level such that the soluble portion from 400 mg. of acetone-desiccated powder was present in each tube. On the other hand, Preparation 7-113A was much more active, so that the soluble part from only 25 mg. was required per tube. In this difference the clue to the unknown factor is present.

It was assumed for purpose of further experimentation that some other constituent of the desiccated tissues was essential to the effectiveness of the inhibitors. With larger amounts of powder more of this was present than in the case of the smaller amounts and consequently greater inhibition was obtained. In other words, one type of "dilution effect," to use the terminology of Schneider and Potter (8), was operating. It appeared reasonable, at least for experimental purposes, to assume further that the coenzyme or dialyzable factor was the responsible and limiting component. At the time the identity of the coenzyme was unknown. However, it was known that manganous or cobaltous salts would reactivate dialyzed enzyme preparations.<sup>1</sup> Consequently, the amount of inhibition obtained with and without added manganous chloride was determined. Unfortunately the above enzyme preparations were no longer available. Instead the two carp preparations indicated in Table IV were used in amounts which produced equal destruction of thiamine.<sup>2</sup> With these the difference in effectiveness of the same concentration of inhibitor is apparent. In the one case 77.5 per cent inhibition is obtained, and with the second only 38.2 per cent. Upon addition of manganous ion so that a final concentration of  $2 \times 10^{-4}$  M of extra manganese is obtained, an increase in inhibition is observed. In Experiment I (Table IV) in which the inhibition is high without addition, the effect of added manganese is small. The actual increase is 8.6 per cent of the control value. In Experiment II in which the control inhibition is 38.2 per cent, the addition of manganese resulted in a larger increase, a value of 53.9 per cent inhibition being obtained instead of 38.2 per cent. The difference due to manganese addition represents an increase of 41.2 per cent above the control value. At the same time, the addition of manganese without the inhibitor had little if any effect upon the enzyme reaction.

<sup>1</sup> Sealock, R. R., and Livermore, A. H., unpublished data.

<sup>2</sup> The authors are indebted to Miss Mary Alice Barber for her assistance in carrying out these experiments.

There is every reason to believe that this effect of the manganese addition is intimately related to the chemical composition of the enzyme preparations and the occurrence of limiting concentrations of essential components. Extensive substantiating evidence for this hypothesis has recently been obtained. It has been found that boiled extracts of acetone-desiccated powder, primarily used for reactivation of inactive dialyzed enzyme, have an effect similar to that observed with manganese ion. When used in conjunction with certain enzyme solutions, these extracts markedly increase the inhibition produced with the thiamine analogues.<sup>3</sup>

TABLE IV  
*Effect of Manganous Chloride on Inhibition*

Additions	Thiamine destroyed	Inhibition
Experiment I. Enzyme equivalent to 25 mg. of Preparation 1-142-III-A		
	<i>micromoles</i>	<i>per cent</i>
None.....	1.83	
1 ml. $25 \times 10^{-4}$ M inhibitor.....	0.43	77.5
1 " $25 \times 10^{-4}$ " " + 1 ml. 0.001 M $\text{MnCl}_2$ .....	0.29	84.2
1 " 0.001 M $\text{MnCl}_2$ .....	1.76	3.7
Experiment II. Enzyme equivalent to 37.5 mg. of Preparation 1-142-I-A		
None.....	1.78	
1 ml. $25 \times 10^{-4}$ M inhibitor.....	1.10	38.2
1 " $25 \times 10^{-4}$ " " + 1 ml. 0.001 M $\text{MnCl}_2$ .....	0.82	53.9
1 " 0.001 M $\text{MnCl}_2$ .....	1.84	-3.0

The same extracts produced no change in the amount of inhibition obtained with still other enzyme preparations. Thus it may be concluded that boiled extracts which contain the coenzyme or dialyzable factor can effect inhibition as does manganous ion. It is, of course, premature at this time to conclude that manganese is the coenzyme, although some evidence to this effect is available.

In contrast to the above results, the addition of manganese did not alter the degree of activation obtained with *m*-amino compounds when these same two enzyme preparations were employed. From this it may be suggested that the activation is the result of a different mechanism. That it does involve the enzyme extract rather than an entirely unrelated chemical effect of the *m*-amino compounds is shown in Table V. In this experiment thiamine was incubated with the same concentration of *m*-

<sup>3</sup> Sealock, R. R., and Sarver, H., unpublished data.

aminobenzyl-4-methylthiazolium chloride ( $5 \times 10^{-4}$  M) with and without the enzyme. It may be seen that an insignificant loss of thiamine occurs when the activator only is present. The 66 per cent loss due to the enzyme

TABLE V

*Effect of m-Aminobenzyl-4-Methylthiazolium Chloride on Thiamine Destruction*

Where enzyme is indicated the equivalent of 400 mg. of mullet viscera, Preparation 7-102A, was used. The same amount of thiamine and buffer was present in all tubes.

Addition	Thiamine destroyed
	per cent
None.....	3
2.5 micromoles benzyl compound.....	1
Enzyme.....	66
“ + 2.5 micromoles benzyl compound.....	93

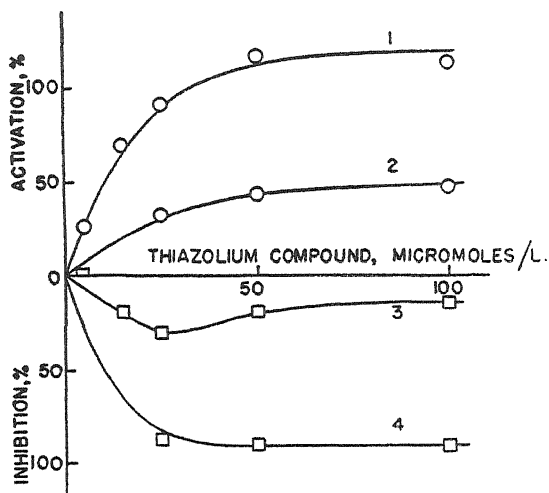


FIG. 2. Effect of benzylthiazolium chlorides on thiamine destruction. The *m*-aminobenzyl-2-methyl compound was used for Curves 1 and 2 and the *o*-aminobenzyl-2-methyl compound for Curves 3 and 4. 25 mg. equivalents of carp Preparation 7-113A were used for Curves 1 and 3 and 400 mg. equivalents of mullet Preparation 7-102A for Curves 2 and 4.

was increased to 93 per cent when enzyme and activator were both present. This constitutes an increased loss of 41 per cent above the control due to the presence of the activator.

A further comparison of inhibition and activation is made in Fig. 2 in which the effect of different concentrations of *o*-amino-2-methylthiazolium

chloride and *m*-amino-2-methylthiazolium chloride on two different enzyme preparations has been determined. In the first place, the increasing inhibition resulting from increased concentration of inhibitor is evident. Likewise by comparing Curves 3 and 4 the striking difference may be seen in per cent inhibition at a given concentration of compound with the two different enzyme preparations. The activation by the meta compound (Curves 1 and 2) increases with increasing concentration, reaching a maximum at about the same concentration which produces maximum inhibition with the *o*-amino compounds. Of further interest is the difference in behavior of the two preparations. In this case the greatest activation is observed with the least weight of powder. With higher quantities of powder represented (Curve 2) very significantly lower activations are obtained. This is quite the reverse of the situation in inhibition and is also suggestive that a different mechanism may be concerned.

#### DISCUSSION

By the use of additional benzylthiazolium analogues of thiamine it has been possible to clarify further the structural components necessary to inhibition of thiamine destruction by the enzyme of fish tissues. Of first importance in the results described here is the additional evidence of the rôle played by the *o*-amino group. Without it, as in the unsubstituted benzyl compound, or with it removed by one position (meta) or two (para), inhibition is not obtained. Since it must not only be present, but also in a definite structural relation to the methylene bridge joining the two rings, it may be argued that the union between enzyme and inhibitor is achieved by this group and one or more additional groups or component parts. Likewise, it may be argued that these two (or three) parts must be in a definite spatial configuration relative to each other.

Of the other positions of the inhibitor molecules it would appear that the 2 and 4 positions of the thiazole moiety need no further consideration. The results obtained clearly demonstrate that there may be a hydrogen at the 2 and a methyl group at the 4 positions, respectively, or the converse with essentially equal inhibition occurring. Since the chemical reactivities of the two carbons are not identical (compare, for example, the behavior of quaternary thiazole derivatives with alkali), equal inhibition would not be obtained with the two different thiazolium compounds if these particular carbons were involved. Such a conclusion is confirmed in part by the definitely weaker inhibition obtained with the 2,4-dimethyl compound. It seems reasonable to assume in this case that the presence of two methyl groups introduces a degree of steric hindrance sufficient to decrease to a measurable degree the binding of the inhibitor to the enzyme in spite of the possible free rotation of the thiazole ring in relation to the

benzyl ring. It may be that the 2,4-diphenyl-substituted molecule on this basis would be even less effective as an inhibitor.

At this time little further evidence is available relative to the other critical groups sharing in the combination of inhibitor and enzyme, but the increase in inhibition obtained by the addition of extra manganese is possibly of real significance in the question of how union occurs. The obvious difference in inhibition obtained with two different enzyme preparations has led to the search for a third component necessary to the enzyme reaction. In the experiment cited here the inclusion of manganese has partially resolved the problem. The addition of manganese in an amount to make the final concentration of extra manganese  $2 \times 10^{-4}$  M increased only slightly the inhibition obtained with one preparation and that obtained with a second preparation to a much greater degree. Or in other words, since the amount of an enzyme reaction is proportional to the concentration of enzyme-substrate complex, the manganese addition in the presence of inhibitor has effectively decreased the concentration of the reaction-producing complex with subsequent calculated increase in inhibition.

On the basis of results of this type, it may be tentatively suggested that manganese is producing its effect by causing a greater degree of combination of inhibitor and enzyme protein and by actually serving as a point of union between the two. By deductive reasoning on the basis of the modern theory of specific competitive inhibition, it may likewise be suggested that manganese is one means of combination of the substrate (thiamine) and the protein of the enzyme. The suggestion is substantiated by the observation, to be described in greater detail elsewhere, that still higher concentrations of added manganese activate and increase the enzymatic destruction of the substrate resulting from certain preparations. That lower concentrations of manganese would produce increased inhibition without appreciably affecting the uninhibited reaction, as shown in Table IV, is not entirely understandable. However, it should be remembered that the affinity constant of the enzyme and inhibitor is approximately 40 times that of the substrate and enzyme. Actually, the discrepancy between the two constants may be even greater than this figure would indicate, for at this date it is impossible to be certain that adequate concentrations of manganese or coenzyme were present in those experiments in which the constants were evaluated.

These results and suggestions are of interest in that unsolved field of the mechanism of enzyme action, but they have also additional significance. For example, with the increasing importance of chemotherapeutic and antibiotic compounds and the recognition of some of these as competitive enzyme inhibitors, the implication is obvious. In those instances in which the compound produces its useful effect by combining with an enzyme

protein through a coenzyme or similar group, which may or may not be a metal ion, as in this case, a deficient or limiting concentration of the coenzyme will cause the inhibitor to be less effective. As these experiments suggest, this is most likely to occur when the inhibitor-enzyme affinity constant is greater than the enzyme-substrate affinity constant. Stated in the reciprocal terms of the dissociation constant, this value for the enzyme-substrate complex must be the larger of the two constants. The fact that the limiting concentration of the essential attaching or binding component may arise as a result of a specific dietary deficiency serves only to emphasize extensive ramifications of the problem. It may be suggested further that in the analysis of drug and enzyme antagonisms the possible function of intermediate linking groups should be evaluated, particularly in relation to the dissociation constants involved.

#### SUMMARY

The previous finding that thiamine destruction by the Chastek paralysis enzyme of fish tissues is inhibited by *o*-aminobenzyl-(3)-4-methylthiazolium chloride has been confirmed. The analogous 2-methyl compound is about equally efficient as an inhibitor, but the 2,4-dimethylthiazolium salt is distinctly less effective.

Further confirmation of the rôle of the *o*-amino group in the inhibition process is evident in the inability of benzyl, *m*-aminobenzyl, and *p*-aminobenzyl derivatives of the thiazoles to inhibit the enzyme reaction.

The degree of inhibition obtained with the *o*-amino compounds at a given concentration varies with the enzyme preparation employed and is usually least with those preparations representing the least amount of enzyme source material. With these the degree of inhibition is increased by the inclusion of extra manganous ion.

The *m*-aminobenzylthiazolium salts not only fail to inhibit the enzymatic destruction of thiamine, but actually cause increased destruction or activation. This increased destruction of thiamine occurs only in the presence of the enzyme extracts and not when thiamine is incubated with the analogue alone.

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# A SYNTHESIS OF ISOTOPIC CYTOSINE AND A STUDY OF ITS METABOLISM IN THE RAT\*

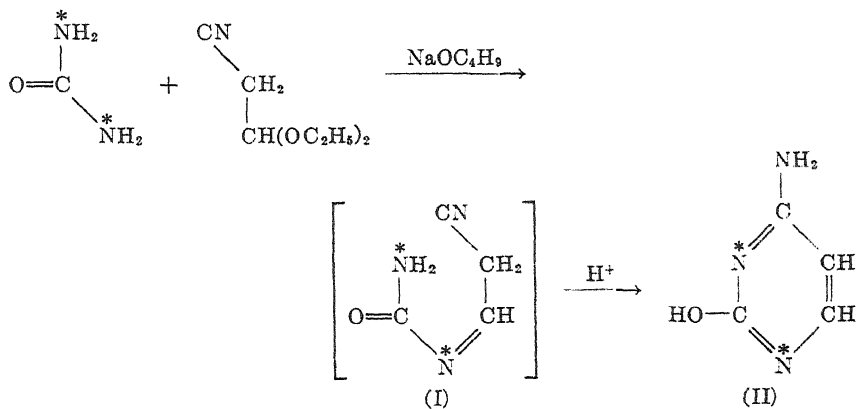
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(Received for publication, October 2, 1948)

Of the five nitrogenous bases known to be present in nucleic acids, the metabolism of isotopically labeled samples of uracil, thymine, guanine (1), and adenine (2) has been investigated. A study of the metabolism of the fifth, cytosine, is the subject of the present investigation. A synthesis has been developed which is suitable for the introduction of isotopic nitrogen into cytosine. Existing syntheses of cytosine suffer from the disadvantage that isocytosine (3) is formed as a by-product, and inferior yields of the desired product result (4).

The synthesis of cytosine described here involves the condensation of urea containing an excess of N<sup>15</sup> (5) with cyanoacetal in the presence of sodium butoxide. The primary condensation product does not exhibit the specific ultraviolet absorption spectrum of cytosine but after brief treatment with dilute acid the typical spectrum of cytosine is observed. The initial condensation probably proceeds via the open chain ureide (I) which would not be expected to show the optical properties associated with pyrimidines (6). On acidification, cyclization to cytosine (II) results in an over-all yield of 56 per cent. This behavior is reminiscent of the cyclization of



\* The authors gratefully acknowledge the use of funds from the James Foundation of New York, Inc., the National Cancer Institute of the United States Public Health Service, and the Office of Naval Research.

cynoacetyl urea to 6-amino-2,4-dihydroxypyrimidine (7, 8) and is consistent with the difficulty experienced in attempting to condense urea with malononitrile (9).

The cytosine obtained was characterized by counter-current distribution (10), by ultraviolet spectroscopy, and by conversion to the picrate, the sulfate, and the ammonium salt.

Labeled cytosine was fed to rats at a level of 160 mg. per kilo of body weight per day. The internal organs were processed for nucleic acids and the various urinary constituents were isolated (Table I). The small amount of dietary cytosine nitrogen found in the nucleic acid pyrimidines (0.22 per cent<sup>1</sup>) is of the same order as that found (0.23 to 0.32 per cent<sup>1</sup>) by Plentl and Schoenheimer (1) following administration of labeled uracil and

TABLE I  
*Feeding of Isotopic Cytosine*

	Atom per cent excess N <sup>15</sup> *	Atom per cent N <sup>15</sup> , calculated on basis of 100 per cent N <sup>15</sup> in cytosine fed
Cytosine (dietary).....	6.00	100
Sodium nucleic acids.....	0.009	0.15
Purine hydrochlorides.....	0.004	0.07
Silver pyrimidines.....	0.013	0.22
Urea.....	0.022	0.37
Ammonia.....	0.017	0.27
Allantoin.....	0.011	0.18
Total urinary nitrogen.....	0.102	1.70

\* Consolidated Nier model No. 21-201, ratio mass spectrometer; average error  $\pm 0.002$ .

thymine. Thus free cytosine, like uracil and thymine, is ineffective as a precursor of nucleic acids in the rat when contrasted with the marked uptake of the purine adenine (13.7 per cent,<sup>1</sup> for an equivalent intake in the diet (2)).

The isotopic nitrogen in the urea and the ammonia shows that at least some complete degradation of cytosine results, and the slightly higher N<sup>15</sup> value for the urea probably signifies that some direct conversion to urea took place. It should be noted that the degradation of cytosine to urea and ammonia was appreciably less than was the case with uracil and thymine (1).

The isotope content of the total urinary nitrogen, which was much higher than that of the isolated constituents, indicated that the cytosine had been extensively absorbed and that a large part was excreted in some less de-

<sup>1</sup> Calculated on the basis of 100 per cent N<sup>15</sup> in the compound fed.

graded form. This is in accord with the data of Mendel and Myers (11) and of Cerecedo (12, 13), who showed that a considerable portion of orally administered cytosine could be recovered in the urine unchanged. The extent of elimination of the nitrogen of dietary cytosine is shown by the data in Table II, where it may be seen that more than four-fifths of the isotope contained in a sample of labeled cytosine was eliminated in the urine within 3 days after its ingestion.

The immediate metabolic precursors of nucleic acid pyrimidines, that is, those more complex than ammonia (14) or glycine (15), are still unknown. A suggestion that oxalacetate may be implicated in this connection has been made by Mitchell and Houlahan (16). The failure of the free pyrimidines to serve as precursors of nucleic acid pyrimidines stresses the

TABLE II

*Elimination of N<sup>15</sup> in Urine of Rat Given 0.906 Mg. of N<sup>15</sup> in Form of 1,3-Labeled Cytosine*

Days after N <sup>15</sup> cytosine feeding	Total N excreted	Atom per cent excess N <sup>15</sup> *	N <sup>15</sup> excreted	Per cent N <sup>15</sup> fed accounted for
	mg.		mg.	
1st.....	645	0.054	0.349	38.4
2nd.....	462	0.048	0.223	62.9
3rd.....	355	0.047	0.167	81.3
4th, 5th.....	832	0.005	0.042	85.9
6th.....	433	0.003	0.013	87.3

\* Consolidated Nier model No. 21-201, ratio mass spectrometer; average error  $\pm 0.002$ .

importance of similar investigations as to the rôle of their ribosides. Such studies are under way in this laboratory.

#### EXPERIMENTAL

*Cyanoacetaldehyde Diethyl Acetal*—The method of McElvain and Clarke (17) was used for the preparation of the cyanoacetal from ethyl- $\beta$ , $\beta$ -diethoxypropionate, which was most conveniently prepared from ethyl orthoformate and ethyl bromoacetate according to Tschitschibabin (18).

*Cytosine Sulfate*—Sodium (2.76 gm.) was dissolved in 90 ml. of anhydrous *n*-butanol in a three-necked flask equipped with a sealed mechanical stirrer and a condenser protected from atmospheric moisture. To the sodium butoxide solution, 7.20 gm. of dry urea and 17.4 ml. of freshly distilled cyanoacetal were added and the whole was refluxed for 2 hours. Mechanical stirring was necessary to prevent bumping due to the deposition of the sodium salt of the intermediate (I). After chilling, the sodium salt (I) was removed by filtration and was washed with chilled butanol and ether. The

filtrate and washings were concentrated to dryness *in vacuo* and the residue was united with the main fraction. The combined solids were dissolved in 150 ml. of hot 2 N  $\text{H}_2\text{SO}_4$ , the hot solution was clarified with Darco, and 2 volumes of hot ethanol were added. Cytosine sulfate (16.8 gm.) crystallized upon cooling.

A sample was recrystallized from a mixture of 1 volume of 2 N  $\text{H}_2\text{SO}_4$  and 2 volumes of 95 per cent ethanol and was dried at  $139^\circ$  over  $\text{P}_2\text{O}_5$  *in vacuo*.

$\text{C}_4\text{H}_5\text{N}_3\text{O} \cdot \text{H}_2\text{SO}_4 \cdot \text{H}_2\text{O}$ . Calculated, S 14.1; found, S 14.1

*Cytosine*—11.8 gm. of cytosine sulfate were dissolved in a minimum of hot water and made alkaline with ammonia and the hot solution was treated with Darco. Glacial acetic acid was carefully added until the pH was 7.0 to 7.5 and, upon cooling, white plates of cytosine deposited (4.32 gm.).

$\text{C}_4\text{H}_5\text{N}_3\text{O}$ . Calculated, N 37.8; found, 37.7 (dried at  $139^\circ$ )

The molecular extinction coefficient, determined on a  $10^{-4}$  M solution in 0.1 M phosphate buffer, pH 6.5, was 6280 with  $\lambda_{\text{max.}} = 267 \text{ m}\mu$ . Counter-current distribution analysis indicated a homogeneous product with partition coefficient  $K = 0.205$  in *n*-butanol-1 M phosphate, pH 6.57 (10). For further characterization a sample of the *ammonium salt* was prepared by crystallization from dilute ammonia.

$\text{C}_4\text{H}_5\text{N}_3\text{O} \cdot \text{NH}_4$ . Calculated, N 43.7; found, 43.5 (dried at  $139^\circ$ )

*Cytosine picrate* was obtained by treating an aqueous solution of cytosine with saturated aqueous picric acid.

$\text{C}_4\text{H}_5\text{N}_3\text{O} \cdot \text{C}_6\text{H}_5\text{O}_7\text{N}_2$ . Calculated, N 24.7; found, 24.9 (dried at  $139^\circ$ )

Counter-current distribution analysis of the picrate showed that excellent separation of cytosine ( $K = 0.204$ ) from picric acid ( $K \cong 9.3$ ) occurred in the system *n*-butanol-1 M phosphate, pH 6.57 (10).

*Isotopic Cytosine*—Analytically pure cytosine containing 6.0 atom per cent excess  $\text{N}^{15}$  (with the isotope in positions 1 and 3) was prepared from urea containing 9.0 atom per cent excess  $\text{N}^{15}$  (5) and cyanoacetal by the above procedure. The only modification introduced was a boric acid trap for recovering ammonia liberated during the reaction. This amounted to less than 2 per cent of the urea used.

*Feeding Experiments*—Three adult Sherman strain male rats with an aggregate weight of 1003 gm. were fed, over a period of 3 days, a total of 480 mg. of cytosine (6.0 atom per cent excess  $\text{N}^{15}$ ) admixed with 177 gm. of moistened Rockland rat diet (complete) to which the animals had been previously accustomed. The daily urine voidings were collected and pooled. The animals were sacrificed on the 4th day. For the isolation of

urinary constituents and of nucleic acids the procedures described (2) were used. The results are given in Table I.

The ultraviolet absorption spectrum of the urine, although it was obscured by considerable non-specific end-absorption, showed a weak maximum in the region of 260 to 275  $m\mu$ , which is not inconsistent with the presence of cytosine. No attempt was made to isolate cytosine because of the small sample.

*Urinary Excretion of Cytosine Nitrogen*—A male Sherman strain rat weighing 329 gm. was maintained for 3 days on a moistened stock diet consisting of 20 gm. of Rockland rat diet pellets per day. On the 4th day, labeled cytosine (40.2 mg.) containing a total of 0.906 mg. of  $N^{15}$  excess was included in the usual diet and thereafter only normal food was offered. The animal's weight remained essentially constant. Total daily urinary collections were made, and care was taken to wash down the residual urine adhering to the glass funnel of the metabolism cage. The total volumes of the combined urine and washings were recorded and total N (Kjeldahl) as well as  $N^{15}$  determinations were made. The data are listed in Table II. The values are probably subject to some small upward revision since the collections cannot be considered as strictly quantitative.

The authors wish to express appreciation to Mr. William Geren and Mr. Eric Godefroi for assistance, to Mr. R. C. Funk, Jr., for the microanalyses, to Mr. Arthur Brandt for the counter-current distribution analyses, and to Miss Alice Angelos and Dr. Harold Beyer for cooperation in the isotope determinations.

#### SUMMARY

A synthesis of cytosine, containing an excess of isotopic nitrogen in positions 1 and 3, from urea and cyanoacetaldehyde diethyl acetal is described.

Evidence is presented that dietary cytosine does not serve as a specific metabolic precursor of nucleic acid nitrogen.

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# A COLORIMETRIC MICROANALYTICAL METHOD FOR ACETATE AND FLUOROACETATE\*

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(Received for publication, June 24, 1948)

The use of lanthanum as a specific reagent for qualitative identification of acetate has long been known (1). The method usually employed (2) involves mixing, in that order, 1 drop of each of the following: (a) suspected acetate sample, (b) 0.01 N  $I_2$ , (c) 5 per cent  $La(NO_3)_3$ , and (d) 1 N  $NH_4OH$ . This results in the formation of basic lanthanum acetate which adsorbs iodine, giving a characteristic blue color. The solution is jelly-like in consistency, is turbid, and the blue color may or may not be distributed homogeneously. Acetate in amounts equal to about 0.2 mg. per ml. of final solution can be detected (2).

It has been found<sup>1</sup> that by heating the mixture smaller amounts of acetate can be detected. A method has been found for removal of interfering inorganic ions ( $Cl^-$ ,  $PO_4^{=}$ ,  $SO_4^{=}$ ,  $Ca^{++}$ , and  $Mg^{++}$ ). By making certain alterations in concentration of reagents, virtually clear solutions can be obtained. Furthermore, the blue color developed can be used as a quantitative measure of the amount of acetate present. The method has been found applicable to monofluoroacetate as well as acetate. While certain difficulties with the method remain to be eliminated, the results obtained thus far seem sufficiently encouraging to warrant this report.

## *Description of Method*

### *Reagents—*

(a) 0.02 N iodine solution. Dissolve 2.54 gm. of reagent grade iodine and 33.2 gm. of reagent grade potassium iodide in distilled water. Dilute to 1 liter.

(b) 2.5 per cent  $La(NO_3)_3$  solution. Dissolve 12.5 gm. of c.p. grade  $La(NO_3)_3$  in 500 ml. of distilled water.

\* The work described in this paper was done under contract between the Medical Division, Chemical Corps, United States Army and the University of Chicago Toxicity Laboratory. Under the terms of the contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

<sup>1</sup> The authors wish to thank Mr. Cecil Nelson, Mrs. Betty Podolsky, and Dr. M. A. Lipton for many helpful ideas and for conducting certain experiments during the development of the method.

(c) 0.10 N  $\text{NH}_4\text{OH}$  solution. Confirm normality by titration against standard acid.

Equal quantities of reagents (b) and (c) are mixed each day to provide a combined reagent containing 1.25 per cent  $\text{La}(\text{NO}_3)_3$  and 0.05 N  $\text{NH}_4\text{OH}$ . The pH value of this mixture must lie between 8.3 and 8.5.

*Removal of Interfering Substances*—The procedure to be described was designed specifically to remove chloride which interferes with color development and calcium, magnesium, sulfate, and phosphate which cause turbidity in alkaline solutions containing lanthanum. The reagents and quantities specified were chosen to remove these substances from a solution used for culturing microorganisms (3). The solution contained originally  $\text{NaAc}$  18.6,  $\text{NH}_4\text{Cl}$  8.62,  $(\text{NH}_4)_2\text{SO}_4$  0.76,  $\text{K}_2\text{HPO}_4$  8.62,  $\text{CaCl}_2$  0.11, and  $\text{MgCl}_2$  0.11 mm per liter. The pH value of the solution when samples were taken for analysis varied from 6.0 to 8.5. Obviously, in the event that different amounts of interfering substances were present, the concentrations of the reagents described below would have to be correspondingly changed.

The procedure which we have used is to put 3.0 ml. of the acetate solution in a 15 ml. centrifuge tube and add the following in the order indicated: (a) to remove chlorides, 1.0 ml. of 0.03 N  $\text{AgNO}_3$ ; (b) to remove excess silver, 1.0 ml. of 0.01 N  $\text{KI}$  (after 2 to 3 minutes have elapsed to allow coagulation of  $\text{AgCl}$ ); (c) to remove sulfate, phosphate, calcium, and magnesium, 1.0 ml. of a solution containing 0.045 N  $\text{Ba}(\text{OH})_2$  and 0.045 N  $\text{Ba}(\text{NO}_3)_2$ . The pH value of the solution at this time should be about 9.5. By making the mixture more alkaline (pH 9.5) than the final solution in which the color is developed, calcium and magnesium, which would cause turbidity, are removed. The solution is then centrifuged or filtered and used as described below.

*Development of Color. Acetate Solutions*—Into a 5 ml. test-tube which can be sealed with a glass stopper or with a rubber tube closed with a glass rod (glass to glass contact) put in order (1) 1.0 ml. of acetate sample containing 80 to 250  $\gamma$  of acetic acid (if a photoelectric colorimeter with about 1 cm. cell depth is to be used) or 200 to 1000  $\gamma$  (if a biological colorimeter of the DuBoscq comparison type is to be used); (2) 2.0 ml. of the combined (1:1)  $\text{La}(\text{NO}_3)_3$ - $\text{NH}_4\text{OH}$  reagent; (3) 1.0 ml. of the 0.02 N iodine reagent. Larger volumes of solution may be employed provided the relative proportions are kept constant and the gas phase is maintained at a minimum.

Seal the tubes and heat in a boiling water bath for 5 minutes. Cool in a beaker of cold water and measure the color developed. Heating time is not critical up to 15 minutes, provided the tubes are well sealed. Fading, caused by loss of iodine, occurs in open or poorly sealed tubes. The color of the developed tubes appears green to the eye. This results from the



superposition of the yellow of excess iodine on the blue of the basic lanthanum acetate-iodine complex.

For measurement of the blue color developed we have employed almost exclusively a Beckman model DU spectrophotometer with rectangular cuvettes of 1 cm. depth, requiring about 3 ml. of solution. A Coleman model 10S spectrophotometer has also been used. With both of these instruments a wave-length of 625 m $\mu$  was selected. When the Evelyn photcolorimeter is used, about 8 ml. of solution are required; we have used Filter 660.

*Fluoroacetate Solutions*—The procedure is the same as that described above except that the amounts of fluoroacetate required are about 3 times those required when acetate is used. Thus use 100 to 400  $\gamma$  of fluoroacetic acid for photoelectric colorimeters. As noted below, with the present procedure larger amounts of fluoroacetate cannot be used satisfactorily.

### Results

In Fig. 1 is presented a graph showing per cent transmittance *versus* the wave-length of light for a solution with color developed as above. A blank employing all reagents, but with distilled water substituted for the acetate solution, was used for establishing 100 per cent transmittance at each wave-length. The curve for the color developed with fluoroacetate is the same except that extinction coefficients differ as noted above.

In Fig. 2 data are presented graphically showing per cent transmittance at  $\lambda = 625$  m $\mu$  *versus* the amount of acetic acid. It will be noted that the procedure described above for removal of interfering substances does not affect the amount of color developed.

In Fig. 2 also are presented data showing graphically per cent transmittance *versus* the amount of fluoroacetic acid at  $\lambda = 625$  m $\mu$ . (Sodium fluoroacetate twice recrystallized and containing less than 0.5 per cent inorganic fluoride was employed.) It will be noted that the curve departs from linearity when more than 400  $\gamma$  of fluoroacetic acid are used. This is discussed below.

### DISCUSSION

We have used the method as described above for measurement of amounts of acetic acid disappearing from cultures of microorganisms. The results, to be reported elsewhere, were indistinguishable from those obtained when acetic acid was distilled and titrated with alkali. The procedure described here was, by far, more rapid and convenient, particularly when large numbers of analyses were to be made.

For the present we can recommend the method described with a previ-

ously prepared calibration curve only when results accurate to  $\pm 5$  per cent are required. If greater accuracy is desired, we recommend simultaneous development of color in tubes containing known amounts of acetate.

It will be noted from Fig. 2 that the proportional amount of color developed decreases when more than 0.4 mg. of fluoroacetic acid is used. This is most probably due to inadequate lanthanum. Increasing the lanthanum concentration, however, leads to increased turbidity and loss of sensi-

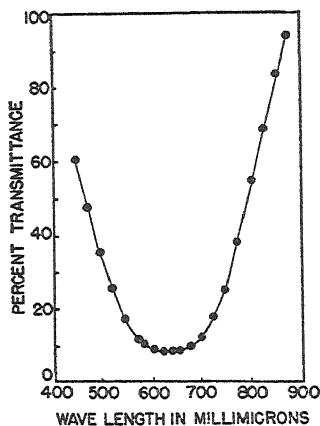


FIG. 1

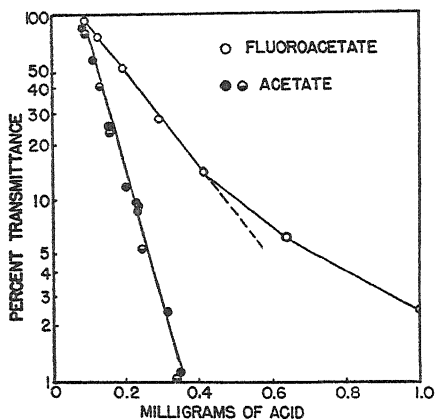


FIG. 2

FIG. 1. Relation of wave-length to per cent transmittance of basic lanthanum acetate-iodine complex. Cell depth 1 cm., solution volume 4 ml., 0.25 mg. acetic acid, Beckman model DU spectrophotometer.

FIG. 2. Relation of amount of acetic acid and fluoroacetic acid to per cent transmittance at 625  $m\mu$  following development of iodine-basic lanthanum complex. O, pure sodium fluoroacetate solution; ●, pure sodium acetate solution; ⊖, acetate from culture solution freed of interfering materials as described in the text.

tivity of the method. Within the limits of color intensities usable with most photoelectric colorimeters the curve is essentially linear. We can only speculate as to the different sensitivities of the method for acetate and fluoroacetate. Possibly the greater acidic strength of fluoroacetic acid ( $pK_A = 3.6$ ) as compared with acetic acid ( $pK_A = 4.76$ ) is responsible. Changing the hydrogen ion concentration of the color-developing mixture leads to undesirable loss of sensitivity, development of turbid solutions, or both.

The specificity of this method for acetic and monofluoroacetic acids has not been independently investigated. Propionates are known to give color (2). Presumably other mono- or polyhalogen derivatives of acetic acid,

*e.g.* iodoacetic acid, would give color. As we have stated above, lactic and pyruvic acids interfere by reducing iodine. Destruction of these by use of oxidizing agents (4) should be as successful in conjunction with this method as in titrimetric procedures.

We have made many attempts to isolate and analyze fluoroacetic acid in tissues and poisoned grain. These have failed universally but the causes are apparently unrelated to the peculiarities of the method described above. That reducing substances such as glutathione, cysteine, etc., were not responsible for the failure was indicated by lack of reduction of the iodine. Analyses for fluorine in the extracts, distillates, and other derivatives from animal tissues indicated that the fluoroacetic acid was not present. This is puzzling in view of the poisonous nature of the tissues of animals poisoned by fluoroacetate (5). A firm binding of fluoroacetate or its conversion to another substance is suggested.

#### SUMMARY

1. A quantitative, colorimetric, microanalytical method for acetic acid (80 to 250  $\gamma$ ) or monofluoroacetic acid (100 to 400  $\gamma$ ) is described.
2. A procedure is outlined for removal of known inorganic ions interfering with color development or measurement.
3. Present limitations of the method are discussed.

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# STUDIES ON GROWTH OF MICE FED RATIONS CONTAINING FREE AMINO ACIDS\*

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(Received for publication, July 16, 1948)

With the announcement by Rose (1) that rats will grow when fed rations containing free amino acids as the sole source of protein, the study of the requirements of experimental animals for individual amino acids was greatly simplified. During the past 10 years Rose and many other investigators have used this method for determining the approximate requirements of the rat for several of the amino acids. Similar studies have been carried out with the chick (2) and the dog (3).

Totter and Berg (4) have shown that the requirements of the mouse for individual amino acids are very similar to those of the rat. They have further shown that D-tryptophan and D-histidine allow less rapid growth than the L forms and that D-lysine fails to support growth. Bauer and Berg (5) extended this work to show that the mouse can utilize both the D and the L forms of methionine and phenylalanine, but only the L forms of valine, leucine, isoleucine, and threonine. They reported that the rate of growth was not retarded by the removal of arginine from the ration. The amino acid mixture used in their study was patterned after that employed by Rose and Rice (6) for growth studies with the rat, except that the total concentration of amino acids was increased to 33.6 per cent by the addition of extra quantities of both the essential and non-essential amino acids. Moderate growth was obtained on this ration, but it was far below that obtained on a similar diet in which the same level of nitrogen was furnished by intact casein. Slow growth occurred when mice were fed a similar ration containing only the ten essential amino acids as a source of nitrogen.

Woolley (7) has shown that the growth rate of mice fed rations containing acid-hydrolyzed casein is increased when supplemented with intact casein at a level of 2.0 per cent. From these results and extensive work with microorganisms, he postulates the presence of a growth factor, strepogenin, in intact casein.

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from The National Foundation for Infantile Paralysis, Inc., New York.

We are indebted to Merck and Company, Inc., Rahway, New Jersey, for the crystalline vitamins.

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Further work by Woolley (8) has shown that heated crystalline tryptogen, heated horse hemoglobin, crystalline insulin, and whole dried beef liver contain this active principle, but that dialyzed heated egg albumin is inactive. Concentrates of streptogenin have been prepared from tryptic digests of casein and found to be as active as the intact protein. From this work he concludes that, in addition to certain amino acids, preformed peptides are needed for optimum growth of mice.

The basal nitrogen source used by Woolley in these streptogenin studies was acid-hydrolyzed casein. Very little work has been presented in which the effect of streptogenin has been studied when free amino acids were used as a source of nitrogen. The work reported here was done in an attempt to obtain an optimum growth rate for mice fed rations composed of free amino acids, and to determine the cause or causes for the observed differences between the rate of growth of mice fed free amino acids and those fed intact proteins.

#### EXPERIMENTAL

All mice used in these studies were of the Swiss Webster strain; however, during the course of this work it became necessary to use mice from three sources. Series I, II, and III were made with animals obtained from our stock colony, Series IV from the colony of the Veterinary Science Department of the University of Wisconsin, and Series V, VI, and VII from Rockland Farms, New City, New York. Because of the differences in the rate of growth observed in mice obtained from these three sources, a casein control group was always included. The rate of growth of the mice receiving the casein ration was assigned a value of 100 per cent, and growth rates obtained on all other rations within the same series were calculated as a percentage of the growth with casein. Equal numbers of male and female mice were used in the first four series, but only male animals were used in Series V, VI, and VII.

Mice were placed in individual screen bottom cages and were fed the ration and water *ad libitum*. All animals were weighed twice weekly and all studies covered a 21 day growth period.

The composition of the basal amino acid mixture<sup>1</sup> containing 5.8 per cent of the active isomers (hereafter referred to as 5.8 per cent essential amino acids) is given in Table I along with the amount of nitrogen derived from each amino acid and the total nitrogen present. In calculating the nitrogen content of all rations, it was assumed that nitrogen from biologi-

<sup>1</sup> Amino acids used in these studies were purchased from Merck and Company, Inc., Rahway, New Jersey, and from the H. M. Chemical Company, Ltd., Los Angeles, California.

cally inactive D forms of free amino acids could be utilized for use other than direct protein synthesis.

The basal ration contained the following ingredients per 100 gm.: essential amino acid mixture 8.2 gm., Salts IV (9) 4.0 gm., corn oil (Mazola)<sup>2</sup> 5.0 gm., thiamine hydrochloride 0.3 mg., riboflavin 0.3 mg., pyridoxine hydrochloride 0.3 mg., nicotinic acid 1.5 mg., calcium *D*-pantothenate 2.0 mg., inositol 100 mg., sodium *p*-aminobenzoate 100 mg., biotin 0.01 mg., pteroyl-glutamic acid 0.025 mg., choline chloride 0.3 gm., monosodium phosphate 0.63 gm., and sucrose to make 100 gm. Any alterations or additions to the amino acid component of the ration were made at the expense of sucrose. When non-essential amino acids were included in the ration, the concentration of glutamic acid was varied to establish the desired nitrogen level. The monosodium phosphate was added to compensate for the phosphorus present in an 18 per cent casein ration, for which the salts mixture was designed.

### Results

Previous work from this laboratory (10) has shown that a slow rate of growth is obtained when mice are fed a synthetic ration in which all nitrogen is furnished in the form of the ten essential amino acids, when supplied at a level  $1\frac{1}{2}$  times the amount shown in Table I. Since casein must be fed at a level of 12 per cent (1.9 per cent nitrogen) to provide a fairly rapid rate of growth of mice, and since the amino acid ration contained only 1.4 per cent nitrogen, the effects of supplementation with non-essential amino acids to provide this higher level of nitrogen were determined.

It was shown that a reduction in the content of the amino acid mixture from 8.7 per cent essential amino acids (Ration 7-I)<sup>3</sup> to the 5.8 per cent essential amino acids level (Ration 1-I) gave a slight increase in the growth rate of the animals. A further increase was produced by increasing the nitrogen level from 1.0 to 1.9 per cent by the addition of L-glutamic acid (Ration 2-I). The substitution of glycine for part of the glutamic acid, without changing the nitrogen level, further increased the growth rate (Ration 3-I). Since previous work had shown that higher levels of glycine are toxic to mice, the effects of further increases in this component were not studied. The addition of 5.4 per cent casein (Smaco) to the basal amino acid mixture (Ration 4-I) gave a significant increase in the rate of growth; however, this increase was not significantly greater than that given by the addition of glycine and glutamic acid alone. An increase in the fat content

<sup>2</sup> Fortified with vitamins A and D from oleum percomorphum.

<sup>3</sup> The arabic numeral indicates the ration number in Table II; the roman numeral indicates the series number.

(Ration 5-I) or the addition of 1.0 per cent liver extract<sup>4</sup> (Ration 6-I) failed to stimulate growth further (*cf.* Table II for rations).

Since the amino acid rations were rather hygroscopic, it was felt that the addition of cellulose (Cellu flour) might be of benefit. Therefore all rations in Series II to VII inclusive contained 10 gm. of Cellu flour added to every 100 gm. of ration, unless otherwise stated. A comparison of groups on Rations 22-V and 30-V shows that cellulose increased the rate of growth of

TABLE I  
*Composition of Amino Acid Mixture\**

Amino acid	Active form needed	Form used	Amount needed per 100 gm. ration	Amount fed per 100 gm. ration	Per cent nitrogen
	<i>per cent</i>		<i>gm.</i>	<i>gm.</i>	
Lysine HCl·H <sub>2</sub> O†.....	1.0	L	1.0	1.37	0.192
Tryptophan.....	0.2	DL	0.4	0.40	0.054
Histidine HCl·H <sub>2</sub> O†.....	0.4	L	0.4	0.54	0.103
Phenylalanine.....	0.9	DL	0.9	0.90	0.076
Leucine†.....	0.8	L	0.8	0.80	0.086
Isoleucine†.....	0.5	DL	1.0	1.00	0.107
Threonine†.....	0.5	"	1.0	1.00	0.118
Methionine.....	0.6	"	0.6	0.60	0.056
Valine†.....	0.7	"	1.4	1.40	0.168
Arginine HCl.....	0.2	L	0.2	0.25	0.065
Total.....	5.8		7.7	8.26	1.025

\* The composition of this mixture is the same as that used by Rose (1) for the rat, with the exception of tryptophan. The level of tryptophan was doubled because of the poor utilization of the D form by the mouse. The levels of lysine, histidine, and arginine were increased to correct for the H<sub>2</sub>O and HCl.

† The D form is inactive for the mouse.

mice fed amino acid rations. However, no significant increase in the growth rate was obtained with mice receiving cellulose added to the rations containing intact casein as the sole source of nitrogen (compare groups on Rations 21-VI and 20-VI). Since the synthetic rations are water-soluble, the inclusion of bulk may increase the physiological availability of its various components; also, by reducing the hygroscopic nature of these rations, the cellulose may increase palatability. When animals are fed synthetic rations containing this added ingredient, they remain sleek and clean, whereas those not receiving cellulose soon appear wet and unkempt in appearance.

<sup>4</sup> Preparation 1432, a water-soluble, alcohol-insoluble extract of whole liver kindly supplied by the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.



Since it was found that an elevation of the nitrogen level of the ration by the addition of glycine and glutamic acid gave a significant increase in the rate of growth, an attempt was made to determine to what extent further additions of these two amino acids would alter the growth rate. Upon examination of the growth rates obtained on Rations 13-III, 16-III, 22-IV, and 24-IV, it may be seen that the rate of growth increased directly with the concentration of glutamic acid up to 11.6 per cent (2.5 per cent nitrogen). Any further increase in the glutamic acid concentration depressed growth, both in the presence and absence of glycine. Thus it appears that the mouse is unable to tolerate glutamic acid at levels higher than 11.6 per cent with this ration.

That the mouse is unable to utilize fully inorganic nitrogen in place of nitrogen derived from glutamic acid is indicated with Ration 25-IV. The replacement of part of the glutamic acid with ammonium chloride and sodium bicarbonate resulted in a definite retardation of growth.

The addition of 2.0 per cent L-glutamine<sup>5</sup> in Ration 14-III gave slightly improved growth over mice fed supplements of glutamic acid and glycine alone (Ration 13-III). However, when the level of nitrogen was increased to 2.5 per cent with glutamic acid and glycine alone (Ration 16-III), the rate of growth was significantly greater than that obtained with the glutamine ration. Since at 2.0 per cent nitrogen only a slight increase in the rate of growth was provided by glutamine, no attempt was made to study the effects of this component on growth at a higher level of nitrogen.

Womack and Rose (11) have recently reported growth results obtained with rats fed a new amino acid mixture, designated by them as Mixture XXIII-b. This mixture differs from the one given in Table I in that arginine is absent, the levels of the essential amino acids have been increased, and glycine, DL-alanine, DL-serine, L-cystine, L-tyrosine, and DL-aspartic acid have been added. They reported that the addition of proline, arginine, or glutamic acid to this mixture resulted in a significant increase in the rate of growth of rats; however, neither proline nor glutamic acid was as effective as arginine.

When this amino acid mixture was used with mice (Rations 18-III and 19-III), it was observed that the addition of glutamic acid and arginine produced a greater gain in weight than did arginine alone. However, the addition of either component alone did not allow as rapid a growth as was obtained with Ration 15-III, which contained only the 5.8 per cent essential amino acids mixture plus glycine, cystine, and glutamic acid. The addition of a higher level of glutamic acid to Mixture XXIII-b (Ration 27-IV), to bring the total nitrogen to 2.5 per cent, again failed to provide as great a rate of growth as was provided by Ration 22-IV.

<sup>5</sup> Kindly supplied by Dr. F. M. Strong of this laboratory.

TABLE II  
*Ration Compositions and Growth Data*

Ration No.	Composition of ration*	Total per cent N	No. of mice	Gain per day for 21 days							Average growth, per cent of growth on casein
				Series I	Series II	Series III	Series IV	Series V	Series VI	Series VII	
				gm.	gm.	gm.	gm.	gm.	gm.	gm.	
1	5.8 E. A. A.	1.0	27	0.18	0.29	0.23					43-51
2	5.8 " + 8.7 G. A.	1.9	10	0.21							
3	5.8 E. A. A. + 4.9 G. A. + 2.0 G.	1.9	10	0.30							
4	5.8 E. A. A. + 5.4 casein	1.9	10	0.33							
5	5.8 E. A. A. + 8.7 G. A. + 15 corn oil	1.9	10	0.14							
6	5.8 E. A. A. + 8.7 G. A. + 1.0 Lederle liver Preparation 1432	2.0	7	0.19							33-47
7	8.7 E. A. A.	1.4	22	0.12	0.27	0.18					
8	8.7 " + 4.52 G. A.	1.9	5	0.16							
9	16.1 casein	2.1	21		0.57	0.54					
10	5.8 E. A. A. + 6.8 G. A. + 2.0 G.	2.0	20		0.38			0.50			
11	8.7 E. A. A. + 3.4 G. A. + 1.0 G.	2.0	14		0.33						58
12	11.6 E. A. A.	2.0	14		0.13						23
13	5.8 " + 0.2 Cys. + 6.8 G. A. + 2.0 G.	2.0	7			0.35					65
14	5.8 E. A. A. + 0.2 Cys. + 2.8 G. A. + 2.0 G. + 2.0 Glut.	2.0	7			0.39					72
15	5.8 E. A. A. + 0.2 Cys. + 4.4 G. A. + 2.0 G. + 1.0 A.	2.0	7			0.41					76
16	5.8 E. A. A. + 0.2 Cys. + 11.5 G. A. + 2.0 G.	2.5	7			0.44					82
17	8.7 E. A. A. + 0.2 Cys. + 3.42 G. A. + 1.0 G.	2.0	7			0.36					67
18	Mixture XXIII-b + 0.73 A.	1.7	7			0.28					52

TABLE II—Continued

Ration No.	Composition of ration*	Total per cent N	No. of mice	Gain per day for 21 days							Average growth, per cent of growth on casein
				Series I	Series II	Series III	Series IV	Series V	Series VI	Series VII	
				gm.	gm.	gm.	gm.	gm.	gm.	gm.	
19	Mixture XXIII-b + 0.73 A. + 2.0 G. A.	1.9	7			0.33					61
20	19 casein	2.5	19				0.44	0.69	0.82	0.80	100
21	19 " (no Cellu flour)	2.5	4						0.79		96
22	5.8 E. A. A. + 11.6 G. A. + 2.0 G.	2.5	15				0.37	0.49	0.50		65
23	5.8 E. A. A. + 15.4 G. A.	2.5	6				0.28				64
24	5.8 E. A. A. + 15.4 G. A. + 2.0 G.	2.9	11				0.21	0.39			44
25	5.8 E. A. A. + 8.7 G. A. + 2.0 G. + 1.0 NH <sub>4</sub> Cl + 1.7 NaHCO <sub>3</sub>	2.5	5				0.24				55
26	5.8 E. A. A. + 8.7 G. A. + 2.0 G. + 1.1 A.	2.5	6				0.29				63
27	Mixture XXIII-b + 0.73 A. + 8.3 G. A.	2.5	6				0.19				43
28	5.8 E. A. A. + 6.9 G. A. + 2.0 G.	2.3	7					0.46			67
29	5.8 E. A. A. + 4.6 G. A. + 2.0 G. + 5.0 casein	2.5	4					0.55			80
30	5.8 E. A. A. + 11.6 G. A. + 2.0 G. (no Cellu flour)	2.5	6					0.42			61
31	8.7 E. A. A. + 6.2 G. A. + 2.0 G.	2.5	6					0.51			74
32	5.8 E. A. A. + 11.6 G. A. + 2.0 G. + 3.1 NaHCO <sub>3</sub>	2.5	4					0.49			71
33	5.8 E. A. A. (0.2 L-Try.) + 11.6 G. A. + 2.0 G.	2.5	3					0.48			70
34	5.8 E. A. A. + 0.3 M. + 11.3 G. A. + 2.0 G.	2.5	3					0.52			75
35	5.8 E. A. A. + 1.1 A. + 8.6 G. A. + 2.0 G.	2.5	3					0.50			72

TABLE II—Continued

[illegible]

TABLE II—*Concluded*

Ration No.	Composition of ration*	Total per cent N	No. of mice	Gain per day for 21 days							Average growth, per cent of growth on casein
				Series I	Series II	Series III	Series IV	Series V	Series VI	Series VII	
				gm.	gm.	gm.	gm.	gm.	gm.	gm.	
48	5.8 E. A. A. + 2.0 G. + 7.3 G. A. + 0.4 Al. + 1.0 Ty. + 1.0 As.	2.5	5							0.59	74
49	5.8 E. A. A. + 2.0 G. + 10.9 G. A. + 0.4 Al.	2.5	5							0.53	66
50	5.8 E. A. A. + 2.0 G. + 10.8 G. A. + 1.0 Ty.	2.5	5							0.50	63

\* All figures are per cent of the ration. Abbreviations of components indicate the following: A. = L-arginine, A. H. C. = acid-hydrolyzed casein, Al. = DL-alanine, As. = L-asparagine, Cys. = L-cystine, E. A. A. = essential amino acids, G. = glycine, G. A. = L-glutamic acid, Glut. = glutamine, L. L. = lyophilized liver, M. = DL-methionine, S. = DL-serine, Try. = DL-tryptophan, Ty. = L-tyrosine.

Why this amino acid mixture (Ration 27-IV), compounded from the same lots of amino acids as those in Ration 22-IV, gave such poor results is rather difficult to explain. Since the amino acids in Mixture XXIII-b are present in a different ratio to each other than those in the 5.8 per cent essential amino acids mixture, the poor growth obtained may be due to an amino acid imbalance. Thus, even though the amino acids are provided in quantities sufficient for the physiological demands of the body, they may not be present in a ratio necessary to provide optimum utilization. Because a relatively poor rate of growth was obtained with the rations of Bauer and Berg (5), in which twenty amino acids were fed, quantity alone was shown to be insufficient for optimum growth. Also, when properly supplemented acid-hydrolyzed proteins or amino acid mixtures corresponding in composition to complete proteins are fed to mice, only moderate growth is obtained; whereas, if two proteins, each one deficient in a different essential amino acid, are fed together, good growth will result. It therefore appears that imbalance affects only the utilization of free amino acids or short chained peptides and is without significant effect if intact proteins are fed.

Another possible cause for the lowered rate of growth obtained with animals fed free amino acids is the relatively high concentration of D isomers in the amino acid mixtures. Even though Bauer and Berg (5) determined the availability of both the D and L forms of the essential amino acids, it

was assumed that if the D forms failed to support growth they were merely non-utilizable for direct protein synthesis and were non-toxic to the host. Although a comparison of growth between mice fed the L forms of the amino acids and those fed the racemic mixtures would be limited at the present time, due to the difficulties involved in obtaining pure samples of many of the L forms, an investigation of this nature would clarify the rôle of the D-amino acids in nutrition.

With rations containing 2.0 per cent nitrogen, an increase in the rate of growth was noted when the level of essential amino acids was lowered (Rations 10-II and 11-II). When the nitrogen level was increased, however, these differences were no longer apparent, since Ration 31-V provided as great a total gain as did Ration 22-V. However, mice receiving the higher level of the essential amino acids grew at a retarded rate for the first 10 days, and then gained in weight more rapidly than did the group on Ration 22-V during the final 11 days. Thus, even at the higher level of nitrogen, no benefit was derived by increasing the level of essential amino acids. All further studies were made with the 5.8 per cent essential amino acid level.

Although these synthetic rations contained a high concentration of free acids, partial neutralization was without benefit, since the addition of sodium bicarbonate (Ration 32-V) failed to increase the rate of growth.

An increase in the level of either methionine or arginine (Rations 34-V and 35-V) was without beneficial effect. Furthermore, a comparison of Rations 22-VI and 37-VI showed that no significant increase in the rate of growth was obtained by additional amounts of both methionine and arginine. Thus, since no further stimulation of growth could be obtained by either increasing the levels of the essential amino acids or further increasing the level of glutamic acid, the effects produced by the addition of various non-essential amino acids were studied.

A significant increase in the growth rate was obtained by replacement of part of the glutamic acid with DL-serine, DL-alanine, L-tyrosine, and DL-asparagine (Ration 39-VI). The addition of 0.5 per cent DL-serine alone retarded growth either in the presence or absence of added arginine and methionine. The addition of DL-asparagine provided a significant increase in the growth rate, even in the presence of serine (Ration 41-VI). However, this increase in the growth rate was less than that provided by the addition of all four of these non-essential amino acids.

The differences in the rates of growth of mice fed Rations 20-VI, 22-VI, and 39-VI are shown in Fig. 1. It should be noted from these growth curves that, although the total growth of mice fed Ration 39-VI is somewhat less than that of mice receiving the same level of nitrogen from intact casein, the slope of the curve for Ration 39-VI from the 7th to the 14th day

is comparable to that of the casein curve for the same period of time. The greatest difference in growth on these two rations occurred from the 1st to the 7th day. If a factor such as streptogenin is directly essential for growing

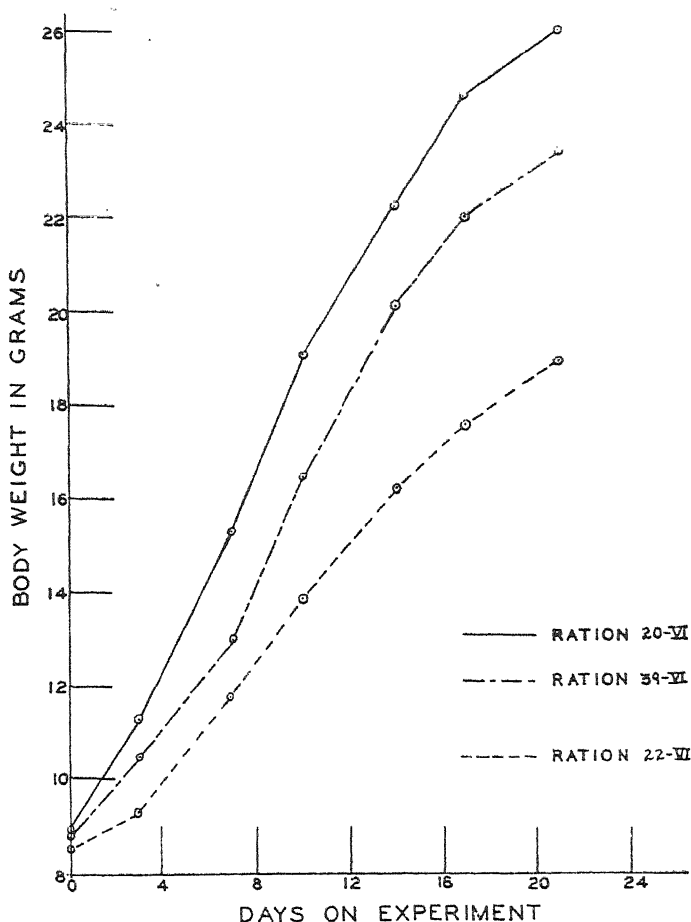


FIG. 1. Growth of mice fed rations containing casein (Ration 20-VI), sixteen amino acids (Ration 39-VI), or the ten essential amino acids plus glycine and glutamic acid (Ration 22-VI).

mice during the 1st week only of a growth experiment, no significant increase in the rate of growth would be obtained by adding non-essential amino acids. An examination of growth curves obtained with mice fed only the 5.8 per cent essential amino acids mixture showed an actual weight loss during the first 3 days, after which the animals gained weight until, on the

7th day, they had attained their original starting weight. However, a definite increase in the rate of growth during this critical period has been obtained by the addition of non-essential amino acids. It would thus seem that this amino acid ration did not provide an initial rate of growth comparable to that of casein because, rather than being deficient in a growth factor such as strepogenin, it is merely less palatable and therefore the mice required a longer period of time to become adapted to it.

Although Totter and Berg (4) have shown with casein hydrolysates that mice are unable to utilize fully the D form of tryptophan, no work has been reported to date on the ability of the mouse to utilize D-tryptophan when fed rations compounded with pure amino acids. A comparison of Ration 33-V with Ration 22-V shows that mice will grow as well on a ration containing 0.2 per cent L-tryptophan as on a ration containing twice this amount of DL-tryptophan. Comparison of Rations 39-VI and 40-VI indicated retarded growth results with mice fed only 0.2 per cent DL-tryptophan rather than 0.4 per cent. Thus it is shown, in agreement with the findings of Totter and Berg, that the mouse is unable to utilize fully for growth the D form of this amino acid. Also, since comparable growth was obtained with 0.2 per cent L- or 0.4 per cent DL-tryptophan, no evidence was present that would demonstrate any toxicity of the D form of this dietary component. Womack and Rose (11) reported preliminary studies that indicate that no level of DL-tryptophan permitted as satisfactory growth of rats as did the same level of the L isomer.

When Ration 39-VI was fed to a second group of mice (Ration 39-VII), the growth obtained was found to be comparable to the casein control group (Fig. 2). Thus it would appear that these animals were able to adapt themselves more readily to the synthetic ration and therefore to grow at a rate comparable to the casein controls.

Under the conditions of these experiments, it is shown that the removal of either serine alone, serine, tyrosine, and asparagine, or serine, alanine, and asparagine decreased the growth rate below the level obtained with Ration 39-VII.

Woolley has reported that the tripeptide serylglycylglutamic acid possessed partial strepogenin activity for microorganisms (12). It is of interest to note that the presence of free serine, glycine, and glutamic acid are required for maximum growth of mice. It is therefore possible that the growth responses with strepogenin concentrates reported by Woolley resulted from the addition of these three amino acids.

As further evidence that strepogenin is not directly essential for growth of these mice, results obtained with Rations 42-VII, 43-VII, 44-VII, and 45-VII are presented.<sup>6</sup> Woolley has reported (8) that casein and liver are

<sup>6</sup> The acid-hydrolyzed casein and the dialyzed, heated egg albumin were kindly supplied to us by Dr. L. M. Henderson of this laboratory.



good sources of streptogenin, whereas dialyzed, heated egg albumin and acid-hydrolyzed casein are devoid of this factor. However, when these four nutrients were added individually to the basal amino acid ration, the growth obtained was not in proportion to the streptogenin concentration.

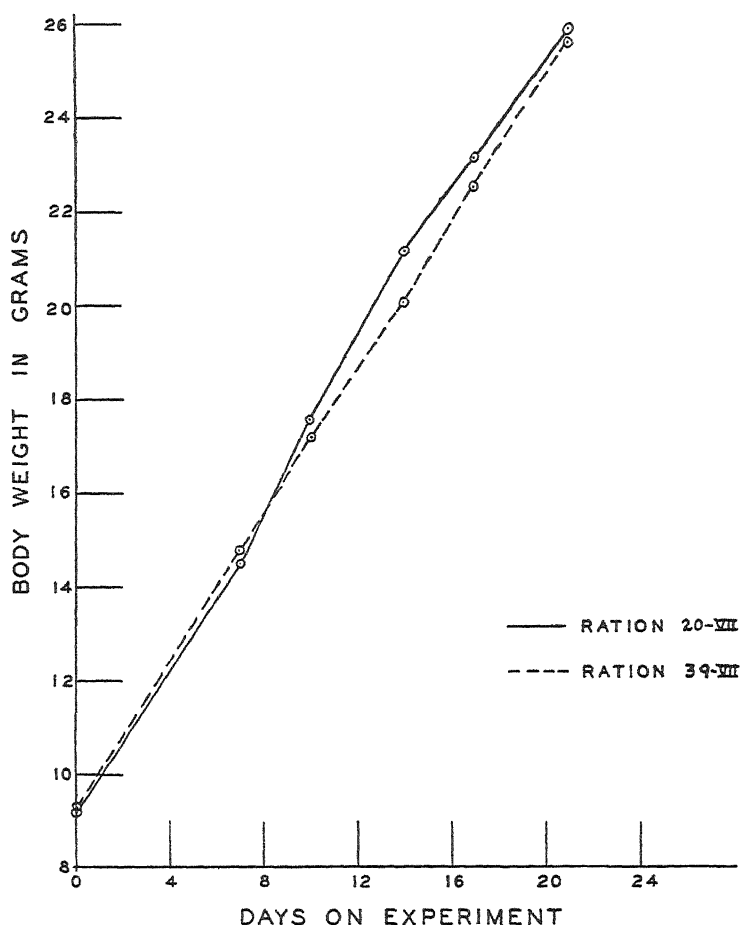


FIG. 2. Growth of mice fed rations containing casein (Ration 20-VII) or sixteen amino acids (Ration 39-VII).

Thus, Ration 42-VII provided a greater weight gain than did either Ration 43-VII or 44-VII. However, none of these four supplemented rations supported as good a rate of growth as did Ration 39-VII, which contained only free amino acids as a source of nitrogen. Furthermore, no beneficial effects were noted when intact casein was added to properly supplemented acid-hydrolyzed casein (Rations 46-VII and 47-VII).

It has been shown from these experiments that amino acid rations, when properly compounded, will provide a rate of growth closely approaching that obtained with casein. Although Ration 39-VII was found to be comparable to casein for growth, this same ration failed to provide completely comparable growth in Series VI. However, it is logical to assume that still further modifications or additions to Ration 39-VII will increase the rate of growth until no differences are noted between animals fed rations compounded with amino acids and those fed intact proteins.

#### SUMMARY

Experiments have been conducted to compare the growth of mice fed casein with mice fed rations containing free amino acids as the sole source of nitrogen.

A ration containing sixteen amino acids properly balanced has been found to support growth closely approaching that obtained with casein.

The addition of intact proteins, rich in streptogenin, failed to improve growth over that obtained with proteins lacking in this growth factor. No improvement in growth resulted upon supplementation of acid-hydrolyzed casein with intact casein. Furthermore none of these rations provided as great a weight gain as did the ration containing only the sixteen amino acids.

The effects of some of these amino acids on growth and possible reasons for the poor growth obtained on previous amino acid mixtures are discussed.

A stimulation of growth provided by the addition of cellulose to these amino acid rations is described.

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# REDUCTION OF IRON IN THE HUMAN STOMACH\*

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(Received for publication, July 24, 1948)

It has been shown that the oral administration of ferrous compounds, as compared with ferric salts of iron, resulted in somewhat more rapid absorption of iron from the gastrointestinal tract (results of studies with radioactive isotopes (1)), and more rapid hemoglobin formation in anemia due to iron deficiency (2). From these results and from the fact that ferrous compounds in general are much more soluble than the corresponding ferric compounds, the theory arose that ferric compounds might be reduced in the intestines to ferrous forms and that this change was essential for absorption. It seemed clear that iron might be reduced in the large intestines, and to a lesser extent in the small intestines, through the action of hydrogen sulfide and other products of bacterial action (3). Histological evidence, however, suggested that iron might be absorbed in the upper small intestine, although no quantitative evidence of the reduction of iron salts there had been presented. New light was thrown on this matter when it was suggested by Tompsett (4) that iron might be reduced by foods in the stomach. He supported this view with demonstrations that iron might be reduced by certain foods when treated with pepsin-hydrochloric acid solutions. While his results were not quantitative and the dipyriddy reaction used was not, as we have shown, well adapted for this purpose, the findings were of great interest. Because of the important bearing that reduction of iron in the stomach and small intestines might have on iron absorption and metabolism, it was decided to carry out studies on human and animal subjects to determine where and to what extent iron was actually reduced *in vivo*.

Since no suitable method existed for determining ferrous and ferric iron in our materials, it was necessary to develop one. This method is described in an earlier report (5). Also reported there are results showing the reduction of iron by various types of foods in artificial gastric digestion.

The present report deals with the reduction of ferric iron present in or added to foods when these are digested in the human stomach.

## EXPERIMENTAL

The subjects were ten apparently normal medical students. Tests were made in the morning on the stomach of a fasting subject, or about 5 hours

\* This work was supported by a grant from the University Research Board of the University of Illinois.

after the last meal. A Rehfuß tube was administered and gastric residuum was removed by aspiration. If any traces of food were present, the stomach was washed out with water. The foods to be tested were then ingested in the ordinary manner. In some cases, foods were given without added iron. In other cases, ferric chloride was given to supply iron in varying amounts of 0.2, 2, 4, 20, or 100 mg. In some cases, 50 ml. portions of gastric contents were removed every 30 minutes for from 1 to 2 hours. In other cases, the stomach was emptied completely at a given time, as at 30 minutes or 1 hour. Specimens were filtered and analyzed immediately, according to the method previously described (5). Filtration was carried out through glass wool. In a few cases, when much pigment was present, the specimens were filtered through acid-washed Celite, which had been shown not to affect the quantity or state of oxidation of the iron present. The results are stated as total amounts of iron and percentages of ferrous iron in the total soluble iron. pH measurements were made with a glass electrode. In certain cases,  $E_h$  values were also determined with a platinum electrode.

#### DISCUSSION

The results are given in Table I.

The main purpose of these experiments was to show whether, by using a number of common foods, reductions of iron would occur in the human stomach, comparable in character to those we observed in artificial gastric digestion. In general, reduction values were found to be somewhat higher than those observed with similar foods *in vitro*.

The results in those cases in which samples of the gastric content only were taken represent the relative proportions of ferric and ferrous iron present in the stomach at the time the sample was removed. In the majority of the cases the stomach was completely emptied and the results then represent the total soluble ferric and ferrous iron present in the stomach at the time of emptying. The figures do not indicate, of course, how much of the iron that had already left the stomach was in the reduced form, nor, therefore, just how much of the total ingested iron was reduced. By emptying the stomach at different times in different experiments, a somewhat more complete picture was obtained.

Iron present in foods or added to a variety of foods was found to be reduced in the human stomach to a significant degree. Foods containing ascorbic acid were particularly effective. Thus, orange juice gave values as high as 77.8 per cent, tomatoes as high as 91.6 per cent, and peas 20 per cent reduction of added iron. Boiled potatoes gave values as high as 52.8 per cent, probably in part due to the ascorbic acid which they contain. Whole wheat bread gave values as high as 38.4 per cent and white bread as much as 59.2 per cent reduction. High protein foods such as meats gave values as high as 53.5 per cent.

TABLE I

*Reduction in Human Stomach of Iron Ingested with Various Foods*

The iron added was in the form of ferric chloride.

Ex- peri- ment No.	Food	Amount given	Iron added	Digestion period	Volume gastric contents	pH	Total soluble iron	Ferrous iron
		gm.	mg.	min.	ml.		γ	per cent
1	Whole wheat bread, dried	30	0	60	50	1.68	230	17.3
			0	90	50	1.45	170	17.6
2	White bread, dried	30	0.2	60	50	1.63	130	30.7
3		30	0	30	50	2.25	120	54.1
			0	60	50	1.78	135	59.2
			0	90	50	1.70	120	29.2
4		30	0.2	30	50	1.70	170	35.3
			0.2	60	50	1.50	140	28.5
5		30	2.0	60	94	1.50	498	16.9
6		30	2.0	60	95	1.50	418	31.8
7		30	20.0	60	100	1.48	1,400	23.2
		ml.						
8	Orange juice	250	0.2	45	50	3.10	70	57.1
9		250	20.0	30	96	3.00	2,928	77.8
10	Tomatoes, minced	250	0.2	30	50	2.50	60	91.6
		gm.						
11	Dried beef, muscle meat	20	0.2	30	50	3.65	115	30.4
			0.2	60	50	2.50	400	42.5
			0.2	90	50	1.80	275	30.9
12	Ground round steak, fat-free	100	0	60	50	3.50	150	36.6
			0	85	50	1.95	90	33.3
13		100	0	60	200	3.30	600	36.6
14		100	0.2	60	50	2.98	130	46.1
			0.2	90	50	1.62	135	29.6
15		100	2.0	60	156	3.30	592	50.0
16		100	2.0	60	260	3.30	728	53.5
17		100	20.0	60	250	2.90	5,000	49.0
18		100	100.0	60	205	3.05	10,250	10.0
19		100	100.0	60	153	3.00	10,710	14.2
20	Cooked liver	100	0	60	83	2.80	398	37.5
21	White potatoes, boiled	162	0.2	60	50	1.98	30	50.0
22		150	20.0	60	130	1.90	3,445	52.8
23		150	100.0	45	140	3.10	7,000	6.0
		ml.						
24	Whipping cream	200	2.0	60	150	3.90	660	13.6
		gm.						
25	Peas, canned	100	0	40	50	1.70	95	36.8
26		100	0.2	30	50	1.70	250	20.0
			0.2	60	50	1.39	100	20.0
27	Whole egg, hard cooked	181	4	30	114	3.20	13	100
28		155	4	30	84	1.60	52	70

TABLE I—*Concluded*

Ex- peri- ment No.	Food	Amount given	Iron added	Digestion period	Volume gastric contents	pH	Total soluble iron	Ferrous iron
		gm.	mg.	min.	ml.		γ	per cent
29	Egg white, hard cooked eggs	173	4	20	83	3.15	33	90
30		180	0	18	12	7.10	0	
31		177	0	20	36	2.95	0	
32		161	4	30	73	3.50	15	100
33		96	4	20	150	7.45	490	59
34	Egg yolks, hard cooked eggs	80	4	20	63	3.55	168	36
35		100	4	20	57	3.60	111	69
36		38	4	30	130	1.90	55	0
37		39	0	30	200	3.80	44	57
38		39	4	30	217	3.40	153	41
39		36	0	30	100	3.80	2	0
		ml.						
40	Homogenized milk	250	0.2	60	34	1.80	61	38.2
41		250	20.0	30	183	3.90	3,202	0
42		500	4	30	103	3.40	0	
43		500	4	30	116	4.25	131	39
44		400	4	20	107	5.20	259	17
45		500	4	30	260	4.28	158	0
46		500	4	30	297	5.30	617	5.5

The results with milk and eggs were somewhat irregular. Thus with milk no reduction was noted in certain cases, while in other experiments reductions of around 39 per cent were attained. It seems possible that added iron combines with the phosphates and phosphoprotein of milk and becomes soluble only after certain degrees of acidity and of protein digestion are reached, after which reduction then becomes possible. Because milk has a strong buffer action, the development of such an acidity may be slow. However, even though an appreciable amount of soluble iron is formed, the amount of reduction may be low because the milk protein does not have a high reducing power. It has been shown (3) that iron fed with casein is less completely reduced in the intestines than when fed with egg albumin or meat proteins.

The exceptional behavior of egg yolk was demonstrated by Tompsett (4) in *in vitro* experiments. He found no reduction of iron added to egg yolk. In certain of our experiments on the human stomach, we found no reduction of the iron of egg yolk or of the iron added to egg yolk. In other cases, however, an appreciable reduction of egg yolk iron or of added iron occurred. Similar results were obtained with whole eggs, while egg white

alone always gave fairly high reducing values. Evidently the iron of egg yolk, as well as iron added to egg yolk, is quite firmly bound. Nevertheless, some release and reduction of such iron are possible in the human stomach.

If iron is administered on the so called empty stomach, it meets with a gastric residuum, averaging about 50 ml. in volume and with a pH usually between 2 and 3. That iron added to such a residuum may be appreciably reduced is shown by the results in Table II. The normal gastric residuum may, therefore, contribute toward the reduction of iron in the human stomach.

That ascorbic acid, as well as proteins and protein digestion products, plays a rôle in iron reduction is clear, not only from the results here presented but also from the work outlined in our previous paper (5). In this

TABLE II

*In Vitro Reduction of Iron with Natural Gastric Residuum*

Incubation was at 37°; 1 mg. each of iron was added.

Residuum			Incubation time	Total soluble iron found	Iron reduced
Volume	pH	$E_h$			
<i>ml.</i>		<i>mv.</i>	<i>hrs.</i>	$\gamma$	<i>per cent</i>
12	2.45	+484	2	336	75.0
66	1.6	+554	1	781	25.0
37	2.3	+461	2	427	37.5
30	4.87	+328	1	341	18.0
35	2.5	+408	1	340	76.5
35	1.89	+435	1	516	20.0
65	2.7	+391	3½	437	35.9

earlier report, the rôle of the formation of complexes of iron with certain food constituents was also considered and shown to be an important limiting factor. It is probable, for example, that the low results on iron reduction sometimes obtained are related to the tying up of much of the added iron with the proteins, phosphates, and other constituents of foods. Tompsett (4) suggested that the failure of egg yolk to reduce iron in his experiments was due to phospholipides.

Within the limits of 0.2 to 20 mg., the amount of iron added did not much affect the percentage of reduction. Thus one subject showed 46 per cent reduction with 0.2 mg. of iron and 49 per cent with 20 mg. of iron on a meat diet. With 100 mg. of iron, there was a decrease in the per cent iron reduced but some increase in the total amount of reduced iron. The iron naturally occurring in such food as cooked ground steak or liver gave values for percentage reduction similar to those obtained with added iron, indicating again that values for reduction depend upon oxidation-reduction

potentials of the mixtures rather than on the amount of some reducing substance present in very limited amounts. Observations on certain foods are still too limited in number to draw definite conclusions and it is not yet clear what all of the reducing substances involved may be.

Oxidation-reduction potentials were calculated in a number of cases. On gastric contents, following milk ingestion, values of +256 to +321 were obtained. With whole boiled eggs, figures of +212 to +356 were obtained. Egg white and egg yolk gave results within the same range. These results were slightly lower than those obtained in our *in vitro* experiments but agreed with them in that they were all below the figure of +747, representing the oxidation-reduction potential of the  $\text{Fe}^{+++}\text{-Fe}^{++}$  system as compared with the standard hydrogen electrode; so that an appreciable reduction of any ferric ions present would be expected in all cases. Further work is necessary. It has, however, been definitely established that reduction of iron by common foods does occur in the human stomach to a very significant degree. It seems clear that this may greatly enhance the opportunity for absorption of iron in the upper small intestines because of the much greater solubility of the ferrous, as compared with the ferric, compounds. This work emphasizes the possibility that gastric reduction may be a primary mechanism in facilitating iron absorption by the body.

#### SUMMARY

Studies have been made in human subjects on the intragastric reduction to the ferrous form of ferric iron present in, or added to, ingested foods. Very appreciable reductions occurred. Thus a number of common foods such as breads, meats, and fruits gave iron reductions as high as 50 to 90 per cent. Milk and eggs gave somewhat irregular results. Ascorbic acid, proteins, and protein digestion products appear to participate in the reduction of iron. The formation of insoluble complexes of ferric iron with proteins, phosphates, and other substances is a limiting factor. Further studies are needed to evaluate more precisely the importance of gastric reduction of iron in relation to the gastrointestinal absorption of this element. The gastric residuum has appreciable iron-reducing properties.

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# BIOSYNTHESIS OF URIC ACID LABELED WITH RADIOACTIVE CARBON\*

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(Received for publication, September 7, 1948)

Sonne, Buchanan, and Delluva (1, 2) have recently identified some of the precursors of the carbon atoms of uric acid in the pigeon. We have had occasion to repeat and extend their experiments during an investigation of the suitability of the pigeon as an agent for the biosynthesis of uric acid labeled in various positions with  $C^{14}$ . This paper presents data on the yields of several different kinds of labeled uric acid obtained after injecting suitably labeled precursors and also provides information on the rôle of the methylene carbon of glycine<sup>1</sup> in the synthesis of uric acid.

## EXPERIMENTAL

Young pigeons were injected subcutaneously or intraperitoneally ten times at 1 hour intervals with  $C^{14}$ -labeled compounds, the total activity injected varying from approximately 0.4 microcurie in the form of glycine to 20 microcuries as formate or bicarbonate. The total quantity injected varied from 0.02 to 0.4 mm depending upon the compound. The excreta were collected during 24 hours following the first injection, except in Experiment 1 in which the pigeon died after 12 hours. In this latter experiment the bird was kept in a large desiccator through which air was passed slowly. This arrangement allowed some rebreathing of respired air and retarded the rate of loss of labeled carbon dioxide. In the other experiments the birds were kept in open cages under a hood.

The uric acid was isolated and purified by the method of St. John and Johnson (3). To determine the distribution of  $C^{14}$  in uric acid, the degradation procedures of Buchanan *et al.* (1) were employed. The data on the distribution and yield of  $C^{14}$  in uric acid formed after injection of labeled bicarbonate, formate, and carboxyl-labeled and methylene-labeled glycine are summarized in Table I.

\* This work was supported in part by a research grant from the United States Public Health Service and by a contract with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned No. 218 in the series of papers approved for publication. The opinions expressed in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsement of the Department of the Army.

<sup>1</sup> The authors wish to thank Dr. Lawrence W. Tuttle for supplying the labeled glycine used in these experiments.

## DISCUSSION

The data of Experiments 1, 2, and 3 confirm previous results (1, 2) by showing that in the pigeon bicarbonate is a precursor mainly of position 6 in uric acid, formate is the precursor of positions 2 and 8, and the carboxyl carbon of glycine is a precursor of position 4. Experiment 4 shows that the methylene carbon of glycine is incorporated to a large extent into positions 2, 4, 5, and 8. The small activity found in position 6 can probably be attributed to carbon dioxide fixation.

The occurrence of the glycine methylene carbon in several positions in uric acid is somewhat unexpected. Buchanan *et al.* (2) concluded on the basis of their experiments with carboxyl-labeled glycine and the experiments of Shemin and Rittenberg (4) with N<sup>15</sup>-labeled glycine that the methylene carbon of glycine goes mainly into the 5 position. Our results show that in fact the largest part of the methylene carbon is found in this position. But

TABLE I  
*Recovery in Uric Acid of C<sup>14</sup> from Various Precursors*

Experiment No.	Precursor	Per cent of precursor activity in uric acid in positions				Total per cent recovery of C <sup>14</sup> in uric acid
		2 and 8	4	5	6	
1	NaHC*O <sub>2</sub>	0.02	0.55	0.10	2.1	2.8
2	HC*OOH	39.3	0.11	0.42	0.08	39.9
3	CH <sub>2</sub> NH <sub>2</sub> C*OOH	0.0	14.7	1.7	0.37	16.8
4	C*H <sub>2</sub> NH <sub>2</sub> COOH	18.9	9.3	31.8	1.2	61.2

in addition the 2, 4, and 8 positions are strongly labeled. The labeling of the 2 and 8 positions suggests that there may be a conversion of the methylene carbon of glycine to formic acid or to the carboxyl group of acetic acid.

Our results show that the biosynthetic method can be useful for the preparation of uric acid labeled in certain positions. The best preparation was obtained from labeled formate (Experiment 2); approximately 40 per cent of the precursor activity was recovered in the uric acid and 98.5 per cent of this activity was in the 2 and 8 positions. Starting with carboxyl-labeled glycine and labeled bicarbonate, the isotope yields in labeled uric acid were lower (16.8 and 2.8, respectively) and the specificity of the labeling was less (87.5 per cent and 75 per cent, respectively, in the 4 and 6 positions). Nevertheless, uric acid labeled in the 4 or 6 position by this method should be useful for some purposes. On the other hand the uric acid prepared from methylene-labeled glycine is of little value because the product contains the isotopes in too many positions; only 52 per cent of the total activity in the uric acid of Experiment 4 was in the 5 position.

Under the conditions employed in these experiments a great dilution of the labeled carbon was experienced in the conversion of the substrate to uric acid. This was true particularly when small quantities of the more highly active substrates were injected. Thus the dilution was 760-fold for each position in the urate labeled in the 2 and 8 positions (Experiment 2) and 1800-fold for the urate labeled in the 6 position (Experiment 1) as compared to 62-fold and 35-fold in the urates labeled in the 4 and 5 positions, respectively (Experiments 3 and 4). Actually the dilution can be adjusted to any desired value by varying the total quantity of precursor injected, and it is also greatly influenced by the time allowed for the collection of the excreta. The only real limit on the specific activity obtainable in the uric acid is the intensity of radiation which the bird can tolerate.

#### SUMMARY

Uric acid labeled in various positions with  $C^{14}$  was prepared biologically by the use of pigeons. Satisfactory yields and reasonably specific labeling were obtained in preparations of uric acid labeled in the 2 and 8 positions, 4 position, and 6 position, starting with labeled formate, carboxyl-labeled glycine, and labeled bicarbonate, respectively. The method is not suitable for the preparation of uric acid labeled in position 5 from methylene-labeled glycine, because the isotope appears also in the 2, 4, 6, and 8 positions.

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# THE COMPETITIVE INHIBITION OF THE UREASE-CATALYZED HYDROLYSIS OF UREA BY PHOSPHATE

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(Received for publication, August 31, 1948)

The inhibition of the urease-catalyzed hydrolysis of urea by phosphate has been noted by a number of investigators (1-3), but there appears to be no information available regarding the nature of this inhibitory action. Assuming the validity of the Michaelis-Menten equation (4), it can be shown (5, 6) that for a system containing enzyme, substrate, and inhibitor

$$\frac{1}{v} = \left(1 + \frac{i}{K_e}\right) \left(\frac{K_m}{V}\right) \left(\frac{1}{s}\right) + \left(1 + \frac{i}{K_n}\right) \left(\frac{1}{V}\right) \quad (1)$$

where

- $e$  = total enzyme concentration
- $s$  = substrate concentration
- $i$  = inhibitor                   “
- $p$  = concentration of enzyme-substrate complex
- $q$  =                   “                   “ enzyme-inhibitor                   “
- $r$  =                   “                   “ enzyme-substrate-inhibitor complex
- $v$  = observed rate for a given initial concentration of  $e$ ,  $s$ , and  $i$
- $K_m = s(e - p - q - r)/p$
- $K_e = i(e - p - q - r)/q$
- $K_n = ip/r$
- $V$  = rate where  $i = 0$  and  $p = e$

It follows from equation (1) that when  $1/v$  is plotted (usually as the ordinate) against  $1/s$  a straight line will be obtained with inhibitory action influencing either the slope or the ordinate intercept or both. Thus the type of inhibition may be defined on the basis of the effect of the inhibitory action upon the slope and intercept in the above plot.

In the absence of an inhibitor ( $i = 0$ ) equation (1) reduces to the Michaelis-Menten equation

$$\frac{1}{v} = \left(\frac{K_m}{V}\right) \left(\frac{1}{s}\right) + \left(\frac{1}{V}\right) \quad (2)$$

permitting the evaluation of  $V$  and  $K_m$ . With competitive inhibition, *i.e.*, when both substrate and inhibitor are presumably competing for the same

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† Contribution No. 1238.

reactive sites, we may set  $K_n = \infty$ , transforming equation (1) into

$$\frac{1}{v} = \left(1 + \frac{i}{K_c}\right) \left(\frac{K_m}{V}\right) \left(\frac{1}{s}\right) + \left(\frac{1}{V}\right) \quad (3)$$

From equation (3) it is clear that with competitive inhibition only the slope will be affected, being increased by the factor  $(1 + (i/K_c))$  over that obtaining when  $i = 0$ . Other types of inhibition, *i.e.* non-competitive, "un-competitive" (6), and "quadratic" (6), may be recognized in the order named by a proportional increase in slope and intercept ( $K_c = K_n$ ), an increase in intercept with no change in slope ( $K_c = \infty$ ), and by apparently unrelated changes in slope and intercept ( $K_c \neq K_n$ ).

TABLE I  
*Effect of Buffer Concentration upon Kinetics of Hydrolysis of Urea by Urease*

Experiment No.	Buffer	Buffer concentration	Slope, $m$	Intercept, $b$	$\frac{m}{b}$	$K_c$
I	Phosphate	$M$				
		0.030	8.1	1.27	6.4	0.038
		0.056	11.3	1.28	8.8	0.037
II	"	0.109	22.1	1.26	17.5	
		0.056	11.9	1.49	8.0	0.034
		0.109	19.4	1.53	12.7	0.033
		0.161	25.8	1.62	16.0	0.034
		0.267	52.0	1.39	37.6	
		0.380	69.1	1.71	40.5	
III	Maleate	0.16	7.8	1.70	4.6	
		0.32	7.8	1.80	4.3	
		0.53	7.8	2.30	3.4	
	Glycine	0.16	6.8	1.45	4.6	
		0.32	7.0	1.54	4.6	
		0.53	7.2	1.68	4.3	

In the urease-catalyzed hydrolysis of urea in the presence of phosphate it has been observed (Table I, Fig. 1) that the ordinate intercept of the  $1/v$  versus  $1/s$  plot remains essentially unchanged with increasing phosphate concentration, whereas the slope increases markedly, the increase being approximately linear for the lesser phosphate concentrations. Upon extrapolation to zero phosphate concentration a value of 4.5 was obtained for the slope ( $m$ ), and from the slope-intercept ratio at zero phosphate concentration a value of 0.003  $M$  urea was obtained for the Michaelis constant ( $K_m$ ) of the urea-urease system at pH 7.0 and 25°.

For a case of competitive inhibition one may obtain from equation (3) the relation

$$m = \left(1 + \frac{i}{K_c}\right) \left(\frac{K_m}{V}\right) \quad (4)$$

and, using the extrapolated value of  $m = K_m/V = 4.5$ ,  $K_c$  may be calculated from the relation

$$K_c = \frac{4.5i}{m - 4.5} \quad (5)$$

where  $i$  = micromoles of inhibitor per ml. For values of  $i$  varying from 30 to 160 micromoles of phosphate per ml. an average value of  $K_c = 0.035$  M phosphate at pH 7.0 and 25° was obtained (Table I). That the observed

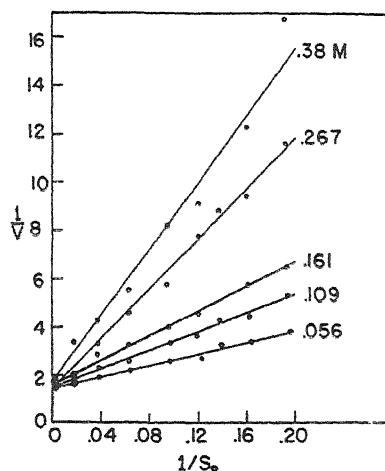


FIG. 1. Effect of phosphate buffer upon the hydrolysis of urea by urease.  $1/v$  in (micromoles of ammonia) $^{-1}$  per ml. per minute,  $1/s_0$  in (micromoles of urea) $^{-1}$  per ml.

inhibition by phosphate is not simply an effect of ionic strength is shown by the markedly different behavior observed with maleic acid and glycine-carbonate buffers adjusted to pH 7.0 (Table I, Fig. 2). Increasing the maleic acid concentration from 0.16 to 0.53 M caused no change in slope and only a relatively small increase in intercept. The increase in slope and intercept noted with increasing concentration of the glycine-carbonate buffer may or may not be significant, since the observed variations are within the limits of experimental error.

It is noteworthy that with the maleic acid and glycine-carbonate buffers a slope-intercept ratio is obtained which is in good agreement with the ratio of 4.5 obtained from the phosphate data upon extrapolation to zero phosphate concentration, and one may conclude that the true Michaelis constant of the urea-urease system at pH 7.0 and 25° is approximately 0.003 M urea.

It is clear that the higher values for  $K_m$  reported previously (7) are a consequence of the hitherto unrecognized competitive inhibitory action of phosphate in the urease-catalyzed hydrolysis of urea. On the basis of computed values for  $K_m$  and  $K_i$  it appears that at pH 7.0 and 25° the inhibitory quotient of phosphate in the urea-urease system ( $K_i/K_m$ ) is approximately 12.

Preliminary experiments with N-butylurea, N-*tert*-butylurea, and N-methylurea have indicated that these substances exert an inhibitory action in the urea-urease system, their effectiveness being in the order named. Present information does not permit definition of the nature of their in-

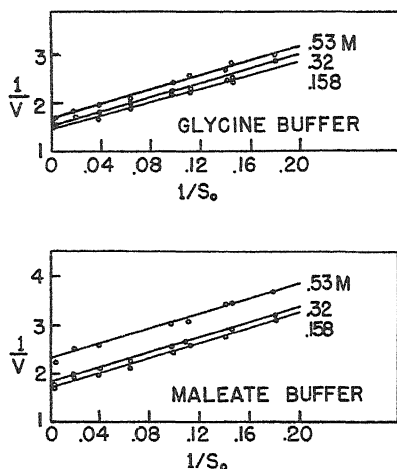


FIG. 2. Effect of maleate and glycine-carbonate buffers upon the hydrolysis of urea by urease.  $1/v$  in (micromoles of ammonia) $^{-1}$  per ml. per min.  $1/s_0$  in (micromoles of urea) $^{-1}$  per ml.

hibitory action, but it does appear that the action is not one of simple competition as was observed in the case of phosphate.

#### EXPERIMENTAL

The procedure used for the determination of urease activity and for the study of the kinetics of the hydrolysis of urea by urease at pH 7.0 and 25° has been described (7). In order to avoid complications arising from the dependence of the specific activity of urease upon the apparent absolute enzyme concentration (7) solutions of thrice recrystallized urease were prepared containing approximately 1  $\gamma$  of protein N per ml., stabilized with hydrogen sulfide (7). These enzyme solutions were 0.01 M in the buffer and were allowed to stand at 25° for 5 hours before use.



The phosphate buffers were prepared from recrystallized dipotassium hydrogen phosphate and potassium dihydrogen phosphate, the maleate buffers by the addition of solid reagent grade sodium hydroxide to a solution of recrystallized maleic acid, and the glycine buffers by the addition of recrystallized sodium carbonate to a solution of recrystallized glycine. In every case, irrespective of the concentration of the buffer, the pH of the solutions was 7.0 at 25°.

A reaction time of 3 minutes was used in all of the experiments herein reported. Control experiments with maleic acid buffers in which the reaction time was varied between 2 and 5 minutes showed only the usual experimental variation. A least squares treatment, in which the data were weighed proportionally to the reaction velocities, was used in computing the slopes and intercepts of the  $1/v$  versus  $1/s$  plots.

#### SUMMARY

The urease-catalyzed hydrolysis of urea has been found to be competitively inhibited by phosphate at pH 7.0 and 25°. The Michaelis constant of the urea-urease system has been found to be approximately 0.003 M urea and the comparable constant defining the phosphate-urease system 0.035 M phosphate.

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# ADAPTIVE ENZYME FORMATION IN THE STUDY OF URONIC ACID UTILIZATION BY THE K-12 STRAIN OF ESCHERICHIA COLI

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(Received for publication, August 13, 1948)

In 1902, Salkowski and Neuberg reported the isolation of D-xylose after the incubation of D-glucuronic acid in a decaying mince (1). Furthermore, it has been noted in numerous plant gums and mucilages that when a single uronic acid and a single pentose are simultaneously present the two are frequently in a homologous series (2), *i.e.* D-glucuronic acid and D-xylose, and D-galacturonic acid and L-arabinose. These findings have led to the hypothesis that the uronic acids are directly decarboxylated to their homologous pentoses (2, 3).

Certain phenomena of adaptive enzyme formation in bacteria appear to provide a tool for the test of this hypothesis, as well as a great many other proposed metabolic steps in bacterial systems. The applicability of the study of adaptive enzyme formation to problems of metabolism has been recently considered by Stanier (4), who has elaborated some theoretical postulates as follows:

"(1) If the dissimilation of a given substance A proceeds through a series of intermediates B, C, D, E, F, G . . . , and if the individual steps in this chain are under adaptive enzymatic control, then growth on a medium that contains A will produce cells that are simultaneously adapted to A, B, C, D, E, F, G . . . (2) If growth on A fails to adapt the cells to a postulated intermediate X, then X cannot be a member of the reaction chain. (3) Growth on E will adapt the cells for F, G . . . but not necessarily for A, B, C, and D."

According to the hypothesis that uronate is decarboxylated to pentose, we are concerned with a single metabolic step. If this mechanism exists, growth in uronate would necessarily require enzymes capable of utilizing further the homologous pentose. It was found in the experiments described below that when uronic acid was utilized as the sole carbon source for growth, depending on the activity of adaptive enzymes for uronic acid, the

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*Escherichia coli* strain studied was nevertheless incapable of utilizing, oxidizing, or fermenting pentose.

We are thus primarily concerned in this report with postulate (2), whose validity we consider highly probable and which excludes a single metabolic step for the production of pentose from uronate. However, the design of an experiment which can possibly demonstrate the existence of a hypothetical pathway by virtue of postulate (1) or exclude it by postulate (2) should clearly demonstrate that the reactions tested are under adaptive enzymatic control. Since the formation of adaptive enzymes is generally accomplished during bacterial growth, the process of bacterial growth must be carefully controlled.

Growth of a bacterial culture on a particular substrate may be possible as a result of one of three distinct types of phenomena: (1) the formation in essentially all the bacteria of enzymes for the utilization of the substrate in response to the presence of the substrate, *i.e.* adaptive enzyme formation; (2) the selection of mutant strains which metabolize the substrate with enzymes capable of being formed during growth on heterologous substrates; or (3) the selection of mutant strains capable of adaptive enzyme formation in response to the particular substrate. In the design of experiments utilizing tests for simultaneous adaptation, the growth techniques used in the preparation of the test bacteria should exclude growth as a function of phenomena (2) and (3), since, otherwise, organisms might have been selected which had acquired several different enzymatic properties, unrelated in the metabolism of the organism, but controlled in some way by a single genetic change. The experimental procedures of Stanier have not excluded this possibility; it has been avoided as much as possible in the experiments reported below by means of the growth techniques described by Monod (5). These involve the continual maintenance of the bacteria in their exponential phase and growth in mixed substrates under conditions minimizing selection while permitting adaptive enzyme formation.

#### *Materials and Methods*

*Bacteria*.—Four strains of *Escherichia coli*, capable of growth on  $\alpha$ -D-galacturonic acid and D-glucuronic acid, were tested for the existence of a diauxie (5) by growth in glucose-L-arabinose mixtures. The most marked diauxie was observed with the K-12 strain of Tatum and Lederberg (6), and this strain of *Escherichia coli* was used in all subsequent studies. It was stored on agar slants and subcultured for use in liquid media of the following composition in per cent: glucose 0.2,  $\text{Na}_2\text{HPO}_4$  1.65,  $\text{KH}_2\text{PO}_4$  0.15,  $(\text{NH}_4)_2\text{SO}_4$  0.2,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.02,  $\text{CaCl}_2$  0.001, and  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.00005. At maximal growth, in cultures aerated by shaking at 37°, viable bacteria numbered 1 to  $2 \times 10^9$  per cc. with glucose as the limiting factor;

on subculture within 24 hours into mineral media of this composition containing glucose, exponential growth began almost immediately. All experiments described below employed bacteria previously subcultured from agar to liquid media in this manner. No significant variations were observed in the growth behavior of the strain throughout the course of the experiments.

*Substrates*—D-glucose and D-galactose were RAL-Kuhlmann products. The Bios Laboratories supplied D-glucuronic and D-galacturonic acids, L-arabinose, and D-ribose; D-xylose was supplied by Hoffmann-La Roche, D-lyxose from Pfanstiehl. I am indebted to Dr. M. Stacey of the University of Birmingham in England for samples of methyl-D-galacturonoside and D-2-desoxyglucose. The acids were neutralized for use.

*Growth Rates*—The increasing optical density of bacterial suspensions aerated by shaking at  $37^{\circ} \pm 0.1^{\circ}$  was determined in the Meunier electro-photometer fitted with a blue filter (5). Under the conditions of the measurement, 1 unit on this instrument was equivalent to about  $10^6$  bacteria per cc. and 0.7  $\gamma$  of dry weight; readings on the instrument were reproducible to 1 unit. The time of doubling of density of these suspensions may be considered to be a mass doubling time (5). It may be noted that the mass doubling time of the K-12 strain in the glucose-containing media used was quite long, *i.e.* of the order of 90 minutes.

*Respirometry*—Bacterial suspensions at appropriate periods of growth, as followed turbidimetrically, were centrifuged and washed twice with either 0.125 M phosphate buffer at pH 6.8 or 0.01 M bicarbonate- $\text{CO}_2$  buffer at pH 7.2. The bacteria were resuspended at one-twentieth the original volume in the appropriate buffer.  $\text{O}_2$  consumption in air or  $\text{CO}_2$  production in 5 per cent  $\text{CO}_2$ -95 per cent  $\text{N}_2$  at  $37^{\circ}$  was tested in Warburg manometers on appropriate substrates.

#### EXPERIMENTAL

*Growth on Media Containing Glucose, Uronic Acid, or Pentose*—The K-12 strain grown on glucose and subcultured to fresh glucose-containing media started exponential growth with a mass doubling time of about 90 minutes. The B strain, described in many studies with bacteriophage (7, 8) and the  $\text{ML}_{3a}\text{L}_a^+$  strain (9) had mass doubling times of 54 and 57 minutes respectively. On galacturonic acid, glucuronic acid, L-arabinose, D-xylose, and D-ribose, the mass doubling times of K-12 cells were about 181, 112, 95, 100, and 176 minutes. Under the conditions given, growth on these substrates, with the exception of glucose (on subculture from media exhausted with respect to glucose), did not begin until after a lag period of about 40 minutes.

*Growth on Media Containing Two or More Substrates*—In mixtures of

glucose and pentose, bacterial growth was a two-step process, *i.e.* the diauxic phenomenon (5), an example of which is given in Fig. 1 by the glucose-xylose growth curve. Thus organisms grown in glucose and transferred to a medium containing these two sugars grew first on the glucose alone until that substrate was exhausted. Despite a prolonged exposure to pentose, adaptive enzymes for pentose were not formed until glucose was exhausted. This is interpreted to mean that glucose inhibits adaptive

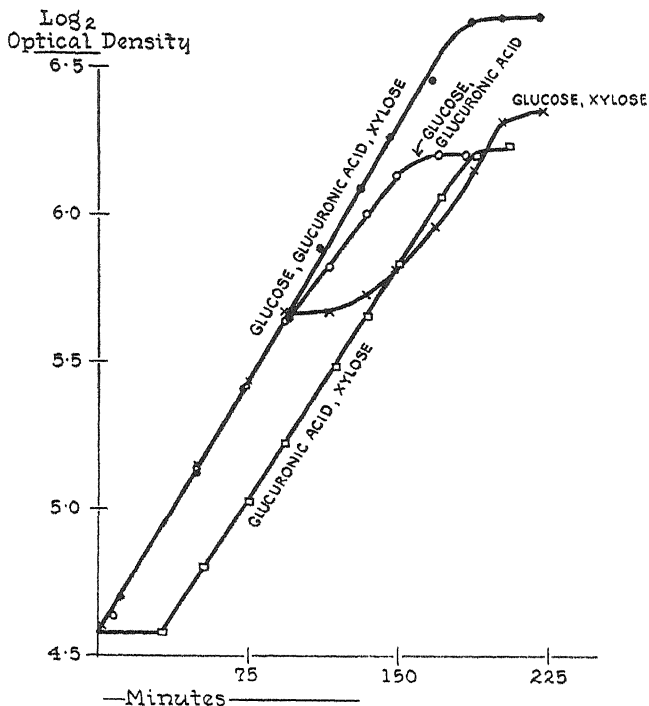


FIG. 1. Growth of K-12 cells at 37° in mineral media containing mixed carbon sources. Each flask contained 150 cc. of mineral medium, 11.2 mg. of each substrate, and 3 cc. of an overnight culture of bacteria in which glucose had been the limiting factor at 2 gm. per liter.

enzyme formation. During the lag period after glucose exhaustion, adaptive enzymes for pentose were produced and growth continued on that substrate. The duration of these lag periods was in the order of L-arabinose, D-xylose, and D-ribose. Although growth on arabinose proceeded exponentially at the conclusion of the lag period, the rate of growth on xylose and ribose increased slowly, apparently until maximal amounts of enzyme were obtained, after which time growth was also exponential. The

time to reach exponential growth was much longer for ribose than for xylose.

In marked contrast to this behavior, a new type of phenomenon was noted in glucose-uronic acid mixtures, as presented in Fig. 1 for the glucose-glucuronate growth curve. Exponential growth occurred until glucose exhaustion; exponential growth then continued immediately at the new rate characteristic of the uronic acid. The position of the rate change, which occurred without appreciable lag period, is identical within the precision of the experimental technique to the cessation of growth on glucose alone. This implies that enzymes for the metabolism of uronic acid had been formed during growth on glucose, although significant utilization of the uronic acid for growth had not occurred.

In mixtures of glucuronic acid and arabinose or xylose, after an initial lag period, exponential growth occurred throughout the growth phase; no diauxie was observable. It was impossible to determine from the growth curves alone, as with the glucuronate-xylose growth curve in Fig. 1, whether growth depended on the utilization of a single substrate at a time or on both together. In growth on a glucuronic acid-ribose mixture, however, the slower growth rate for ribose was established near or at the end of uronic acid utilization.

In contrast to their behavior in a glucuronate-pentose mixture, the bacteria were observed to respond differently to a galacturonate-pentose mixture. The homologous system, galacturonic acid-arabinose, behaved as in the above. However, in the heterologous mixture of xylose and galacturonic acid, a diauxie was obtained in which the bacteria first became adapted and grew on xylose and subsequently on galacturonic acid.

Furthermore, although galacturonic acid when present in glucose-arabinose mixtures eliminated the normal diauxie to yield a continual exponential phase, it did not eliminate the glucose-xylose diauxie. This apparent specificity of galacturonic acid-arabinose relations was not noted with glucuronic acid, since glucuronic acid eliminated the diauxie in the glucose-arabinose case as well as in the glucose-xylose case.

Growth of the bacteria in ternary mixtures under the conditions given in Fig. 1 provides organisms maximally adapted to pentoses. This method of pentose adaptation has been useful in the preparation of ribose-adapted bacteria, since in the absence of glucuronic acid the cells reach maximal ribose activity exceedingly slowly.

*Simultaneous Adaptation As Determined by Estimations of Growth Rate*—Bacteria were grown on glucose, glucose-uronic acid mixtures, or in glucose-pentose mixtures. Within 1 hour of substrate exhaustion as determined by turbidity measurements, uronate-adapted and non-adapted cells were inoculated into media containing pentose, and pentose-adapted and non-adapted cells were inoculated into media containing uronate. The densi-

ties of these cultures grown at 37° were followed. Adapted or non-adapted cells had similar lag periods before the start of growth and similar growth rates on the new substrates. However, since lag periods for adaptation to the new substrate were only of the order of 30 to 40 minutes or less, inability to see small time differences did not completely exclude the possibility that such differences might have existed.

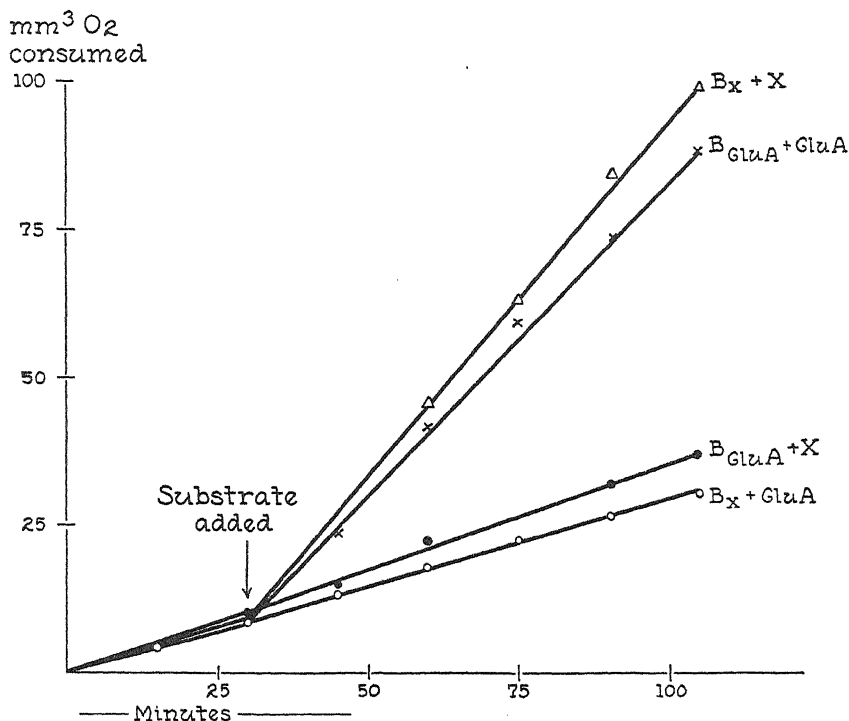
*Other Tests of Simultaneous Adaptation*—In order to test the ability of adapted cells to use new substrates which were previously unutilized, it was necessary to eliminate or minimize the possibility of adaptation in the presence of the new substrate. Three possible techniques were tested: (1) The ability of a bacteriophage to multiply in preadapted K-12 cells plated on agar containing the new substrate as the sole carbon source. It has been shown that phage-infected cells are incapable of enzymatic adaptation (10), a finding consistent with the observation that many types of phage-infected cells appear incapable of synthesizing bacterial constituents (8). Thus, an infected unadapted cell should be incapable of phage multiplication and plaque formation, since a carbon source would be unavailable for phage synthesis. With the kind cooperation of Dr. E. Wollman of the Pasteur Institute, this was tested in the phage  $\phi B_1$ -K-12 system. It was found, however, that  $\phi B_1$ -resistant mutants of K-12 cells were present in such proportions as to obscure plaque formation. This test was not further pursued. (2) It has been found that dinitrophenol (DNP) at  $m/600$  will stop adaptation and in some organisms, at least, permit normal oxidation of glucose (11). It was found, however, that K-12 cells, adapted to uronate or arabinose, were unable to oxidize these substrates in  $m/600$  DNP in 0.12  $M$  phosphate buffer at pH 6.8. This inhibition was reversible; the DNP could be washed out of the cells and the ability of adapted cells to oxidize uronate or pentose was restored. (3) Adapted cells were centrifuged and washed several times in the desired buffer. They rapidly oxidized or fermented the adaptive substrate, as measured in Warburg manometers, whereas unadapted cells did not. It was found that unadapted washed cells resuspended in a phosphate buffer in air frequently became adapted slowly to the uronates or pentose. However, this slow adaptation did not obscure the value of the test of ability to oxidize the new substrate. Washed K-12 cells in a bicarbonate buffer in an atmosphere of  $N_2$ - $CO_2$  were never observed to adapt to the fermentation of the substrates tested.

*O<sub>2</sub> Consumption of Adapted and Non-Adapted Cells*—K-12 cells grown on glucose were completely incapable of oxidizing galacturonic or glucuronic acid, arabinose, xylose, ribose, or 2-desoxyglucose for considerable periods. After shaking in phosphate buffer at pH 6.8 in Warburg vessels for 90 to 120 minutes in the presence of the new substrate, some activity was observable with the uronates. This is interpreted as a very slow adaptation and



is in contrast to the rapid uptake of  $O_2$  on addition of substrate to cells preadapted to uronate. Only 40 to 60 minutes were sometimes adequate for the adaptation to the pentoses in the Warburg vessels. Cells grown in glucose oxidized galactose at a rate about 30 per cent of that on glucose.

It was observed that cells adapted to uronic acid oxidized uronate at a rate only slightly less than that obtained with glucose, but were incapable



[FIG. 2.  $O_2$  consumption of preadapted cells on homologous substrates at  $37^\circ$ . *GluA* and *X* signify glucuronic acid and xylose respectively.  $B_{gluA}$  and  $B_x$  signify glucuronic acid and xylose-adapted bacteria respectively. The vessels contained 2 cc. of washed bacteria in 0.125 M phosphate buffer, pH 6.8, 0.1 cc. of 2 per cent substrate in the side arm, and 0.2 cc. of N NaOH in the center well. The  $QO_2$  of  $B_{gluA}$  and  $B_x$  on *gluA* and *X* respectively were 100 and 125.

of oxidizing any pentose. Conversely, cells adapted to pentose were incapable of oxidizing uronic acid. A typical experiment for the homologous series, glucuronic acid-D-xylose, is presented in Fig. 2. Similarly, no cross adaptation was observed for the series galacturonic acid-L-arabinose.

*Fermentation of Adapted and Non-Adapted Cells*—K-12 cells grown in glucose were incapable of producing acid from the uronates or the pentoses

in a bicarbonate buffer at pH 7.2 in an atmosphere of 5 per cent  $\text{CO}_2$ -95 per cent  $\text{N}_2$ . Cells adapted to uronate liberated 2 moles of acid per mole of uronate, but were inactive on pentose. Conversely, cells adapted to pentose produced 2 moles of acid per mole of pentose and were inactive on uronate. Basal rates in these cells were practically negligible, and adaptation even on prolonged incubation (3 hours) in the substrate in the vessels was never observed.

In a typical experiment, aliquots of 10 cc. of bacteria were added to batches of 200 cc. of medium containing 0.40 cc. of 2 per cent glucose and

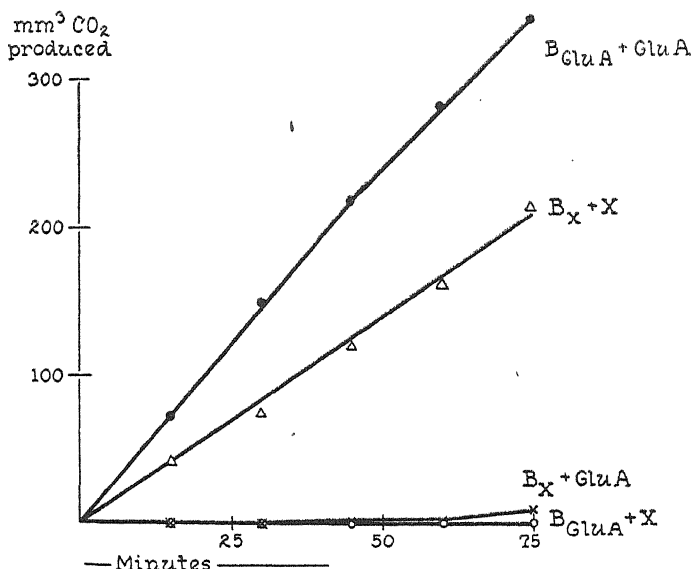


FIG. 3. Acid production of preadapted cells on homologous substrates at 37°. The vessels contained 2 cc. of washed bacteria in bicarbonate buffer, 0.1 cc. of 2 per cent substrate plus 0.1 cc. of bicarbonate buffer in the side arm. The pH of the system was 7.2. The  $Q_{\text{CO}_2}$  of  $B_{\text{gluA}}$  and  $B_x$  on *gluA* and *X* respectively was 170 and 100.

0.40 cc. of 2 per cent glucuronate or xylose. The bacteria were grown to constant density and washed in the centrifuge. They were resuspended in bicarbonate- $\text{CO}_2$  buffer at pH 7.2 and tested on glucuronate and xylose. The results are presented in Fig. 3.

*Specificity of Adaptation*—Adaptation to a single pentose produced cells completely specific for that pentose with respect to both oxidation and fermentation. Cells adapted to galacturonate were inactive to methyl-D-galacturonoside and oxidized galactose at the same rate as did unadapted cells. However, these cells or glucuronate-adapted cells oxidized and fer-

mented both uronic acids. It may be seen from Fig. 4 that glucuronate-adapted cells oxidized galacturonate at about 70 per cent of the rate on glucuronic acid, while galacturonate-adapted cells oxidized glucuronate at about 70 per cent of the rate on galacturonate.

*Intermediate Formation in Uronic Acid Metabolism*—It was observed in many experiments that the initial rates of oxidation or fermentation of uronate as determined in the Warburg vessel were not maximal but soon

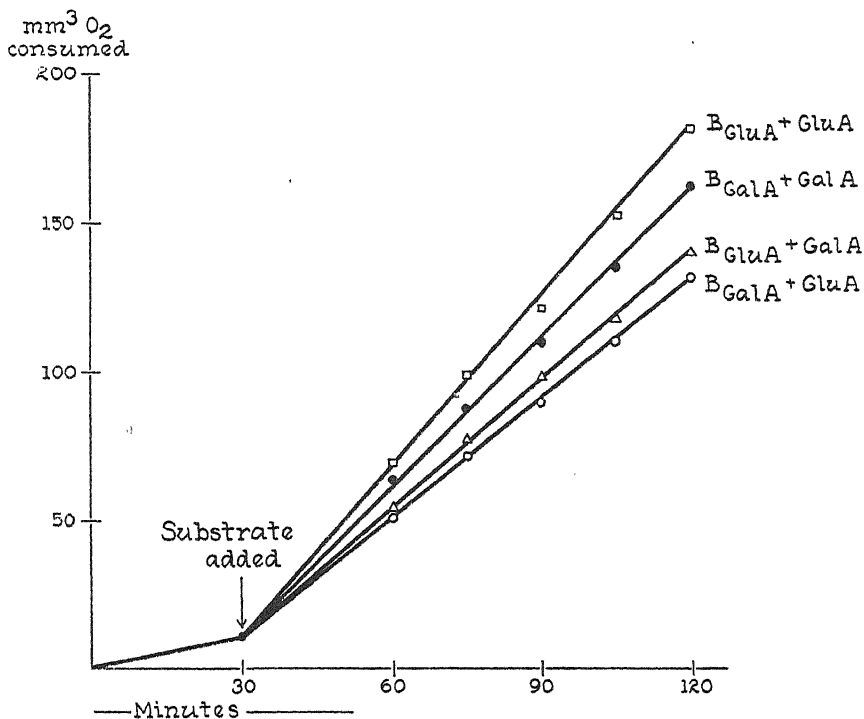


FIG. 4. The specificity of uronate oxidation by uronate-adapted cells. The vessels contained 2 cc. of washed preadapted bacteria in 0.125 M phosphate buffer at pH 6.8, 0.1 cc. of 2 per cent uronate in the side arm, and 0.2 cc. of N NaOH in the center well.

reached maximal constant rate. This effect could be made more marked in two ways: (1) by using starved adapted organisms, or (2) by using organisms adapted to uronic acid in the presence of glucose, which were chosen when relatively small amounts of the uronic acid-metabolizing system had been formed.

In the experiments previously described, adapted organisms were used within 1 hour after the exhaustion of the substrate. When adapted organ-

isms were left in the exhausted medium for about 18 hours, two effects were noted: (1) enzymes for galacturonic acid disappeared in some experiments completely, and (2) the time necessary for the development of maximal rate on uronic acid, *e.g.* the consumption of  $O_2$  of glucuronate-adapted cells on glucuronic acid, was considerably increased. It was found that the addition of a trace of glucose, which was used immediately by starved cells, considerably reduced the time of attainment of constant rate on the uronic acid. An experiment of this type is given in Fig. 5. It may be seen that, with 2 mg. of glucose,  $O_2$  consumption started immediately at the maximal rate;

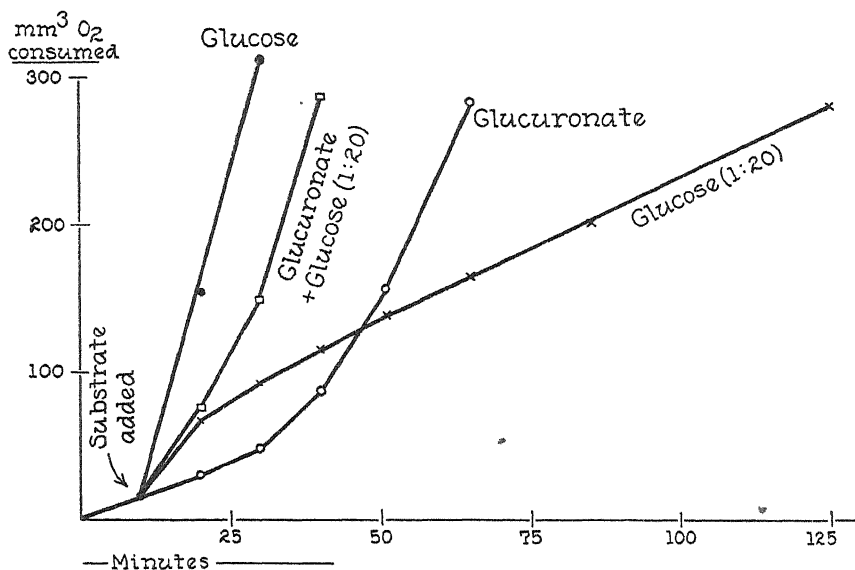


FIG. 5. The priming of uronic acid utilization by glucose in starved adapted bacteria. Glucose, glucuronate, and glucose (1:20) represent the additions of 2 mg., 2 mg., and 100  $\gamma$  of these substrates respectively.

that with 2 mg. of glucuronate, the maximal rate was not obtained after more than 60 minutes. With 100  $\gamma$  of glucose alone, the bacteria resumed their basal rate very quickly. On addition of this much glucose to the glucuronate, the uronic acid was used at the maximal rate in 10 minutes after exhaustion of the glucose. A comparable effect of glucose was not obtained on non-adapted bacteria. This is interpreted to mean that the metabolism of glucose generated compounds which participated in the metabolism of uronate at some stage prior to either oxidation or fermentation.

This experiment does not completely exclude the possibility that the effect of glucose was to stimulate the synthesis of adaptive enzymes or

coenzymes for uronate metabolism. However, increasing rates are found without adaptive enzyme synthesis with cells fermenting uronate in bicarbonate buffer in  $N_2$ - $CO_2$ . In this case, the final rate of acid production obtained with these cells depended on the initial enzyme content of the cells as determined by their mode of preparation (see next section); in the case of oxidizing cells the final rates obtained were identical and independent of the method of preparation, indicating adaptation in the vessels. Thus, increasing rates of acid production in  $N_2$ - $CO_2$  can be interpreted solely as the formation of intermediates from uronates prior to fermentation. This type of data was obtained in experiments described in the next section.

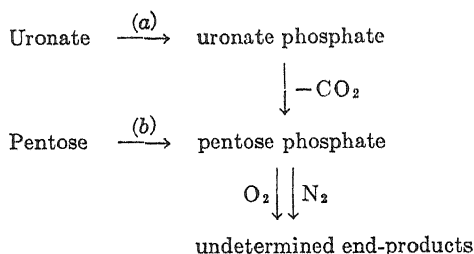
*Rate of Adaptive Enzyme Formation*—The growth curve on glucose-glucuronic acid presented in Fig. 1 indicates an exceedingly rapid synthesis of enzymes for uronate after complete glucose exhaustion or synthesis of enzymes during growth on glucose. Aliquots of bacteria were removed from cultures growing in glucose in the presence of glucuronate or galacturonate and were inhibited from further enzyme synthesis by adding an equal volume of  $M/300$  dinitrophenol. The bacteria were centrifuged, washed to remove the dinitrophenol, and tested for  $O_2$  consumption in air or acid production in  $CO_2$ - $N_2$  on uronate. It was found that small amounts of enzymes were indeed formed for both types of metabolic activity during the glucose phase of growth, which increased in quantity as glucose was exhausted. Analysis of the rates of enzyme production was difficult in oxidizing systems because of the two phenomena described previously which produce increasing rates, (1) adaptive enzyme formation in oxidizing systems, and (2) apparent formation of intermediates in both oxidizing and fermenting systems before constant maximal rates were obtained. The study of rates of fermentation was most practicable for such analysis, since adaptation did not occur in the vessels.

It was found that formation of enzyme for uronate did not begin in the glucose phase until glucose was depleted to a low concentration. Maximal enzyme concentrations were obtained only after growth on the uronic acid alone.

#### DISCUSSION

Since organisms capable of metabolizing uronic acid are incapable of metabolizing the homologous pentoses, it is concluded that L-arabinose is not produced in the metabolism of D-galacturonic acid, and that D-xylose is not produced in the metabolism of D-glucuronate in the K-12 strain of *Escherichia coli*. Although it may be suggested that adaptive growth of bacteria in the uronic acid produced surface effects so that the pentose could not penetrate, the permeability hypothesis to account for the inability of the cells to oxidize or ferment pentose is considered to be unlikely.

However, the data obtained are not as yet inconsistent with the possibility that



According to this hypothesis, enzymes represented by reactions (a) and (b) would be adaptive and of the hexokinase type, *i.e.* uronic acid kinase and pentokinase, and the bacteria already contain enzymes for the oxidation or fermentation of pentose phosphate. Enzymes capable of oxidizing or fermenting pentose phosphate have been found in unadapted *Escherichia coli* strains (12) and have been described for yeast (13, 14). The diploid or haploid strains of yeast tested in the course of these experiments were incapable of growing on pentoses or on uronic acids, suggesting that these yeasts lack only the ability to form the adaptive phosphorylating enzymes for pentose or uronate.

The finding that organisms grown under aerobic conditions have fermentative activities suggests the possibility that these fermentative steps were previously present in the bacteria before adaptation, and that the two types of activity may have only a few steps in common which prepare substrate for the two pathways.

The slow synthesis of enzyme for uronate in glucose-uronate mixtures, until all glucose is exhausted from the medium, indicates that, as in the case of a diauxie, glucose is an inhibitor of adaptive enzyme formation in this system also. In the diauxie, however, inhibition is in most cases essentially complete; in the uronate case, inhibition was not complete. Since the formation of enzyme for uronate was not very great until glucose was exhausted, the utilization of uronate during the last part of the glucose phase of growth could not be sufficient to produce a significantly greater yield on glucose-uronate than on glucose alone.

Nevertheless, the incomplete glucose inhibition is of interest for the further study of adaptive enzymes for uronate and pentose, since the preparation of bacteria with such enzymes can be undertaken under controlled conditions and should not involve strain changes as a result of selection phenomena. Furthermore, the specificity of these bacteria for the quantitative analysis of pentose should prove useful in the study of the pentoses in general.

I wish to acknowledge with gratitude the generous donation of laboratory facilities and the frequent stimulating discussions of the problems described above by Dr. André Lwoff and Dr. Jacques Monod of the Laboratory of Microbial Physiology of the Pasteur Institute. I am indebted to Miss Madeleine Vuillet for her competent technical assistance.

#### SUMMARY

The growth of the K-12 strain of *Escherichia coli* has been studied in media containing glucose, uronic acid, and pentose, separately or in mixtures, as the sole carbon source. The adaptation of cells to the utilization of uronic acid has been demonstrated when the phenomena could be shown not to be the result of selection and when glucose was the primary energy and carbon source. This technique has been further extended to the adaptive preparation of cells maximally active to pentose by growth in pentose, glucose, and glucuronic acid. It has been found that during growth in the glucose phase on glucose-uronic acid mixtures, enzyme for uronic acid was synthesized as glucose was exhausted.

Cells adapted to either glucuronic acid or galacturonic acid could oxidize or ferment both uronic acids. They could not oxidize or ferment D-xylose, L-arabinose, or D-ribose. Evidence was presented that an intermediate, whose formation is accelerated by products of glucose metabolism, is produced before oxidation or acid production can begin on uronic acid. Cells adapted to one pentose could oxidize or ferment that pentose but not other pentoses or uronic acid. It has been concluded that in the K-12 strain of *Escherichia coli* the metabolism of uronic acid does not proceed by the direct decarboxylation of uronic acid to pentose. Another hypothesis for uronic acid metabolism has been presented and discussed.

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# BIOLOGICAL STUDIES WITH 4-AMINO-10-METHYLPTEROYL-GLUTAMIC ACID

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(Received for publication, September 22, 1948)

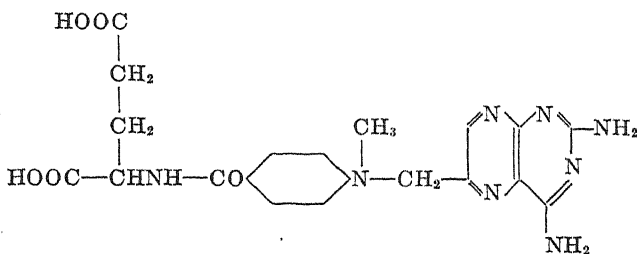
Certain of the various synthetic analogues and derivatives of pteroyl-glutamic acid (PGA) have the property of inhibiting the growth of the test organisms *Streptococcus faecalis* R and *Lactobacillus casei* in the presence of marginal levels of PGA, the inhibition being reversed by raising the concentration of PGA in the culture medium. Such compounds may be termed "PGA antagonists" (1, 2).

An unidentified synthetic product, "methylfolic acid," was the first PGA antagonist to be reported. In one investigation (3) it was prepared by allowing *p*-aminobenzoyl-D-glutamic acid to react with dibromobutyraldehyde and 2,4,5-triamino-6-hydroxypyrimidine under conditions similar to those first described by Angier and coworkers (4). It was found (3) to reverse in a competitive manner the growth-promoting effect of PGA for certain microorganisms. When *p*-aminobenzoyl-L-glutamic acid rather than the D compound was used in the synthetic reaction, the resultant product, crude *x*-methylfolic acid, was found to reverse in a competitive manner the growth-promoting effect of PGA in rats (1), mice (2), chicks (2), and swine (5, 6). During the progress of these observations a number of compounds structurally related to PGA were synthesized and found to behave as PGA antagonists. In general, modification of the pteridine ring of the PGA molecule causes a loss of biological activity or development of an antagonistic effect, although a few compounds have been reported to have a weak PGA-like effect (7, 8). A series of 2,4-diaminopteridines with different substituents on the 6 and 7 positions were studied (9) and were found to include several PGA antagonists for microbial systems (9, 10). One of the most active compounds for *Streptococcus faecalis* was 2,4-diamino-6,7-diphenylpteridine. This compound, however, proved to be inactive when fed to chicks. A related compound, 2-amino-4-hydroxy-6,7-diphenylpteridine, inhibited the growth of chicks but was relatively inactive for *S. faecalis* (11). Pteroylaspartic acid (12) was found to inhibit the utilization of PGA for growth by *Lactobacillus casei*, *S. faecalis* R, and the chick, although it was not effective in this regard for *Escherichia coli* and the rat. Modification of the heterocyclic ring system in PGA by the sub-

stitution of benzimidazole for the pteridine ring (8) resulted in a compound with weak PGA-like activity. However, when in addition to the modification of the ring system the *p*-aminobenzoic acid group of PGA was replaced by its sulfonyl analogue, a PGA antagonist was obtained. The compounds 2-amino-4,7-dihydroxypteridine-6-carboxyl-*p*-aminobenzoylglutamic acid and quinoxaline-2-carboxyl-*p*-aminobenzoylglutamic acid (13) were also shown to be PGA antagonists. A series of N<sup>10</sup>-alkylated pteric acids and derivatives were synthesized (14), and two of these, N<sup>10</sup>-methylpteroic acid and N<sup>10</sup>-methyl-PGA, were found to have a strong effect as PGA antagonists for *S. faecalis* R. A highly active derivative, 4-aminopteroylglutamic acid, which has a bacterial inhibition ratio of about 1, was synthesized (15) and was found to produce marked effects in mice (16), guinea pigs (17), rats (18, 19), chicks (19), and human beings (20). In the present communication the activity of a related derivative, 4-amino-N<sup>10</sup>-methyl-PGA, is reported.

#### EXPERIMENTAL

*Preparation of Antagonist*—The compound used was prepared by Dr. J. M. Smith, Jr., Calco Chemical Division, American Cyanamid Company, using a reaction which has been described previously (4) but in which 2,4,5,6-tetraminopyrimidine and *p*-N-methylaminobenzoylglutamic acid replaced 2,4,5-triamino-6-hydroxypyrimidine and *p*-aminobenzoylglutamic acid respectively. This yielded the product N(4((2,4-diamino-6-pteridyl)-methyl)-N-methylamino)benzoylglutamic acid, 4-amino-N<sup>10</sup>-methyl-PGA (21), which has the structural formula accompanying. The compound had a purity of approximately 88 per cent.<sup>1</sup>



*Microbiological Experiments*—The inhibitory action of the antagonist for microorganisms was studied with *Streptococcus faecalis* R. The medium of Landy and Dicken (22) was used in the assay. The growth period was 16 hours and 10 ml. of medium were used per tube with supplements per 10 ml., as shown in Table I. It was found that the antagonist inhibited the growth-promoting action of PGA in a competitive and reversible man-

<sup>1</sup> Seeger, D. R., Cosulich, D. B., Smith, J. M., Jr., and Hultquist, M. E., *J. Am. Chem. Soc.*, in press.

ner. Over the concentration range studied the inhibitory effect of any level of the antagonist could be reversed and the ratio of antimetabolite to metabolite remained approximately constant if the ratio was calculated as a function of either maximum inhibition or half maximum growth.

*Animal Experiments*—Weanling Wistar male rats were housed in wire-

TABLE I  
*Effect of Pteroylglutamic Acid and 4-Amino-N<sup>10</sup>-methylpteroylglutamic Acid on Growth of Streptococcus faecalis R*

Pteroylglutamic acid	4-Amino-N <sup>10</sup> -methylpteroylglutamic acid	Optical density	Inhibition ratio*	
			Half maximum inhibition	Complete inhibition
<i>mγ</i>	<i>mγ</i>			
0		0.02		
1		0.08		
2		0.17		
3		0.28		
5		0.48		
10		0.77		
10	10	0.77	3	6
10	20	0.54		
10	50	0.09		
10	100	0.02		
10	200	0.01		
100	100	0.31	1	2
100	200	0.04		
100	500	0.01		
100	1,000	0.01		
100	2,000	0.01		
1,000	500	0.54	0.6	1
1,000	1,000	0.09		
1,000	2,000	0.02		
1,000	5,000	0.01		
1,000	10,000	0.01		
10,000	1,000	0.54	0.4	1
10,000	2,000	0.55		
10,000	5,000	0.19		
10,000	10,000	0.02		
10,000	20,000	0.01		

\* Ratio of the amount of antagonist to the metabolite.

floored cages and supplied with feed and water *ad libitum*. A purified basal diet without PGA was used which contained glucose ("cerelose") 72 gm., washed casein (Labco) 20 gm., salt mixture (1) 4 gm., corn oil 3 gm., succinylsulfathiazole 1 gm., and a supplement of vitamins as previously described (1). Four animals were used per group. The rats were weighed

weekly and hematological examinations were conducted at 2 week intervals. The results are shown in Tables II and III.

The results differed quite markedly from those obtained with crude *x*-methyl-PGA; if the animals survived the first few weeks of the experiment they appeared to continue to grow at a normal rate. Even animals which received amounts of the antagonist that were only very slightly below the lethal dosage showed a normal growth response and did not exhibit the

TABLE II

*Effect of Pteroylglutamic Acid and 4-Amino-N<sup>10</sup>-methylpteroylglutamic Acid on Growth and Survival in Rat*

Four rats were used per group.

Group No.	Supplement		Weight increment				No. of animals surviving
	PGA	4-Amino-N <sup>10</sup> -methyl-PGA	2 wks.	4 wks.	6 wks.	8 wks.	
	mg. per kg. diet	mg. per kg. diet	gm.	gm.	gm.	gm.	
1	0.1	0	44	81	115	130	4
2	0.1	0.3	39	71	92	115	4
3	0.1	1.0	36	70	110	141	4
4	0.1	3.0	9				0
5	0.1	10.0					0
6	1.0	0	51	103	140	160	4
7	1.0	0.3	50	101	129	150	4
8	1.0	1.0	34	94	135	176	3
9	1.0	3.0					0
10	1.0	10.0					0
11	10.0	0	56	119	142	190	4
12	10.0	0.3	43	108	137	171	4
13	10.0	1.0	44	90	138	173	3
14	10.0	3.0	6				0
15	10.0	10.0					0
16	100	0	56	114	132	161	3
17	100	0.3	51	96	142	174	4
18	100	1.0	55	89	120	149	4
19	100	3.0	43	91	138	172	3
20	100	10.0					0

type of graded response observed in the deficiency which was induced by crude *x*-methyl-PGA (1). Furthermore the surviving animals were normal in appearance and did not exhibit the unthrifty appearance characteristic of PGA deficiency.

The mortality pattern was of interest. In preliminary experiments in which much higher levels of the antagonist were used animals seldom died before 5 or 6 days and usually all were dead within 1 week, the results

showing no correlation between survival time and dosage. In the experiments presented in Table II most of the animals in the groups which showed toxic effects died at approximately 1 week and only a few rats in such groups survived beyond this time; most of these died within the 2nd week. Any that survived for more than 2 weeks were not subsequently affected. The narrowness of the range of the dosage levels which caused

TABLE III  
*Effect of Pteroylglutamic Acid and 4-Amino-N<sup>10</sup>-methylpteroylglutamic Acid on Hematology of Rats*

Group No.	Supplement, mg. per kilo diet		Hemoglobin				White blood count, cells per c.mm. $\times 10^{-3}$				Differential white cell count, per cent granulocytes			
	PGA	4-Amino-N <sup>10</sup> -methyl-PGA	2 wks.	4 wks.	6 wks.	8 wks.	2 wks.	4 wks.	6 wks.	8 wks.	2 wks.	4 wks.	6 wks.	8 wks.
			gm. per cent	gm. per cent	gm. per cent	gm. per cent								
1	0.1	0	9.1	11.0	13.3	13.3	9.5	10.7	13.5	18.2	45	15	16	19
2	0.1	0.3	9.7	11.0	14.2	12.6	7.9	11.4	11.4	11.6	34	16	17	12
3	0.1	1.0	10.0	11.4	12.8	13.0	9.7	12.8	11.4	15.1	29	17	9	18
4	0.1	3.0	2.4				6.6				0			
5	0.1	10.0												
6	1.0	0	10.2	11.1	14.5	13.8	10.6	11.8	13.8	22.5	30	21	13	31
7	1.0	0.3	9.8	11.4	13.9	14.7	10.5	14.7	17.5	14.7	29	17	17	15
8	1.0	1.0	10.4	11.3	12.9	13.7	9.8	12.8	16.8	15.8	26	20	16	17
9	1.0	3.0												
10	1.0	10.0												
11	10.0	0	9.3	11.1	14.0	13.5	8.8	14.1	15.1	12.9	33	21	14	17
12	10.0	0.3	9.5	10.8	14.0	14.4	14.6	13.1	14.2	16.1	37	31	27	19
13	10.0	1.0	9.7	12.0	13.5	13.2	13.0	14.7	13.1	13.5	28	25	16	20
14	10.0	3.0	4.7				1.4				5			
15	10.0	10.0												
16	100.0	0	10.3	10.6	14.5	14.5	10.2	13.2	14.3	13.7	30	14	14	12
17	100.0	0.3	10.2	11.1	13.2	13.9	11.2	11.4	14.4	16.2	36	20	19	18
18	100.0	1.0	9.9	10.7	13.2	13.4	10.5	13.8	9.9	14.0	28	23	22	19
19	100.0	3.0	7.2	11.7	13.7	13.1	10.4	14.0	11.5	16.3	24	18	12	13
20	100.0	10.0												

moderate inhibition of growth but which were not lethal indicated that the dosage-mortality curve was very steep and consequently the ratio of the LD<sub>100</sub>:LD<sub>0</sub> was unusually small, less than 3.

Attempts to reverse the action of the antagonist, as had been accomplished in the microbiological assay, did not meet with much success. Increasing the antagonist level by a factor of 3.3 above the first level, 3.0 mg. per kilo of diet, which produced toxic signs, resulted in complete mortality.

If the PGA level was then increased 1000-fold, these animals could be protected. However, if the antagonist level was 10 mg. per kilo of diet, which was 10 times the amount that could be tolerated in the presence of 0.1 mg. of PGA per kilo of diet, a 1000-fold increase in the PGA level was without protective effect.

No depression was seen in the blood morphology in the surviving animals. However, shortly before death animals in Groups 4 and 14 were found to have a moderate anemia, leucopenia, and granulocytopenia. Group 19, in which a reversal was affected with PGA, had a normal blood picture.

On gross examination at autopsy the lesions and pathology that have been observed in PGA deficiency and in the syndrome induced by crude *x*-methyl-PGA, including encrustation of the vibrissae with red pigment, alopecia, mouth ulcerations, and unkempt appearance (1), were not gen-

TABLE IV  
*Weight of Chicks Fed Purified Diet Deficient in PGA and with Various Supplements*

Supplement per kilo diet	21 days	28 days
	gm.	gm.
None.....	66 (9)	76 (4)
0.1 mg. PGA.....	133 (10)	201 (10)
0.3 " ".....	165 (10)	265 (10)
0.1 " " + 3 mg. 4-amino-N <sup>10</sup> -methyl PGA.....	121 (8)	175 (8)
10 mg. PGA + 3 mg. ".....	193 (10)	289 (10)

The weight at 0 day was 44 gm., with ten chicks. The figures in parentheses represent the number of survivors.

erally seen. Only a few animals developed a very slight chromodacryorrhea and a moderate gingivitis was occasionally observed. The viscera were normal in appearance and no lesions or pathology were seen other than a slight enteritis.

In an experiment with chicks, a purified PGA-deficient diet was used as described previously (2), except that extracted with hot alcohol casein replaced water-washed casein, *p*-aminobenzoic acid was omitted, and 3 ml. of refined liver extract, 10 U. S. P. injectable units per ml., were added to each kilo of diet as a source of the "animal protein factor" (23). Ten newly hatched New Hampshire chicks were used in each group and the results are given in Table IV. N<sup>10</sup>-Methyl-4-amino-PGA appeared to be less toxic for the chick than for the rat and the slightly growth-slowing effect of a supplement of 3 mg. of the compound per kilo of diet disappeared when the level of PGA was increased.

## DISCUSSION

The effects on rats of the two compounds 4-amino-PGA (16) and 4-amino-N<sup>10</sup>-methyl-PGA were found to be strongly marked and showed little tendency towards reversibility by PGA. In these respects these two compounds may be contrasted with certain other PGA antagonists (1, 2, 14),<sup>2</sup> the action of which disappears when the dietary level of PGA is raised and which show their effect only at comparatively high dosage levels.

Various PGA deficiency signs in rats have been described. When these animals received a purified diet containing a suitable level of a sulfonamide, they were found to develop a syndrome marked by retarded growth, a characteristic blood dyscrasia, alopecia, and a hypoplasia of the bone marrow (24). When crude *x*-methyl-PGA was administered, the development of the syndrome was accelerated and the symptomatology was more severe (1), most animals developing a severe chromodacryorrhea and ulcers of the mouth in addition to the above-mentioned signs. In the present investigation, it was found that 4-amino-N<sup>10</sup>-methyl-PGA, when administered to the rat at a subtoxic level, did not retard growth and caused little alopecia or general unthriftiness. A moderate anemia and leucopenia were sometimes established. Occasionally chromodacryorrhea was seen but it was never very severe.

Attempts to reverse the toxic effects of 4-amino-N<sup>10</sup>-methyl-PGA in the rat by administering PGA have been somewhat more successful than those carried out in mice (15) with 4-amino-PGA. Other studies in the chick embryo<sup>3</sup> indicate that 4-amino-PGA is more toxic than 4-amino-N<sup>10</sup>-methyl-PGA. The effects of the two compounds on the growth of *Lactobacillus casei* and *Streptococcus faecalis* are very similar.

In studies with the rat it was observed that leucopoiesis in the myeloid series was the most sensitive system to PGA deficiency which was observed. This led to the suggestion (1) that PGA antagonists might be used as chemotherapeutic agents in myelogenous leukemia. Farber and his associates (20) have recently published a preliminary report on the effect of PGA antagonists on leukemia in children. They found that temporary remissions could be induced with several of the antagonists and that 4-amino-PGA was the most satisfactory of the compounds tested as judged by clinical, hematological, and pathological observations. However, certain difficulties were encountered because of the toxic properties of this substance. Favorable results have also been obtained by Kracke<sup>4</sup> and Meyer (25) in the treatment of certain cases of leukemia with 4-amino-PGA. Others have recently published similar findings (26).

<sup>2</sup> Unpublished data in this laboratory.

<sup>3</sup> Private communication, Dr. S. Wong.

<sup>4</sup> Kracke, R. R., and coworkers, private communication.

## SUMMARY

1. It was found that 4-amino-N<sup>10</sup>-methylpteroylglutamic acid was a potent antagonist for pteroylglutamic acid (PGA), as measured with rats, chicks, and *Streptococcus faecalis* R.

2. At various levels, the anti-PGA effect of 4-amino-N<sup>10</sup>-pteroylglutamic acid was readily reversed in the assay with *Streptococcus faecalis* R by adding an appropriate excess of PGA, but the toxic effect for rats was reversible by PGA only over a very narrow range.

3. Attention is called to the existence of more than one pattern of signs and symptoms in the syndrome produced by the various PGA antagonists.

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# THE GROWTH RESPONSE OF CHICKS TO ANTIPERNICIOUS ANEMIA PREPARATIONS\*

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(Received for publication, September 23, 1948)

The observation that concentrated liver preparations used in pernicious anemia therapy are highly active in promoting the growth of chicks under standardized conditions was previously reported (1). The present paper gives the results obtained when liver preparations showing different anti-pernicious activity were injected into or fed to the chick.

## EXPERIMENTAL

Day-old, straight run (New Hampshire ♂♂ × single comb white Leghorn ♀♀) cross-bred chicks which were the progeny of hens on Diet B-1 described previously (2) were used in all studies. The chicks were housed in electrically heated batteries with raised screen floors and twelve were used per group. Feed and water were supplied *ad libitum*. Each chick was wing-banded and weighed at the beginning of the experiment and at weekly intervals until termination of the experiments at the end of 4 weeks. The basal ration was the same as that reported in previous studies (3). Subsequent to the finding that the assay was improved by the addition of iodinated casein (4), this material (protamone, 3.07 per cent thyroxine) was included in the basal ration used in Experiments 5, 6, and 7 at a level of 0.03 per cent.

The response of any test group within each experiment is expressed as a percentage of the difference between the positive control (basal supplemented with 3 per cent condensed fish solubles) and the negative control (unsupplemented basal). Thus a comparison of groups within different experiments can be made by reference to the last column of Table I.

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part from funds supplied by the Borden Company, New York, and by the Commercial Solvents Corporation, Terre Haute, Indiana.

We are indebted to Dr. D. V. Frost of the Abbott Laboratories, North Chicago, Illinois, for liver preparations and leucopterin; to Dr. E. D. Campbell of Eli Lilly and Company, Indianapolis, Indiana, for liver preparations; to the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for synthetic folic acid; to the Borden Company, New York, for condensed fish solubles; to Dr. W. R. Graham, Cerophyl Laboratories, Inc., Kansas City, Missouri, for protamone; and to Merck and Company, Inc., Rahway, New Jersey, for crystalline vitamins and vitamin B<sub>12</sub> concentrate.

Studies on the distribution of the factor or factors measured by this growth assay showed that a sample of condensed fish solubles was comparable in potency to whole liver substance (Wilson's) and Viobin 40° liver powder (3). Condensed fish solubles added to the basal ration at a level of 3 per cent served throughout these experiments as the positive control.

The liver extracts used in these studies were purchased from a pharmacy when required with the exception of the experimental preparations which were obtained from the indicated sources.

### *Results*

The results are presented in Table I. It was observed in our initial experiments that the intramuscular injection of 1.0 U. S. P. unit per bird per day of a commercial liver extract (Lilly, reticulogen) produced a rate of growth equal to that of the positive control (1). In Experiment 1 two commercial liver extracts were compared at a lower dosage. The results show that 0.4 unit per bird per day of either preparation was sufficient to cause a full growth response (Lots 3 and 4).

Accurate comparison of potency can only be made at dosage levels causing less than maximal responses. Therefore several dilutions of reticulogen were used in Experiments 2 and 3 to find the dosage at which growth stimulation would be marginal. Dilutions were made to supply 0.02 and 0.01 units per bird per day. The response obtained at these levels was half maximal.

Considerable variation in response to the same dosage was observed between different experiments. For example, 0.4 unit per bird per day gave a response of 118 per cent in Experiment 1 and a 78 per cent response in Experiment 2 (Lots 3 and 7). A level of 0.1 unit showed a 95 per cent response in Experiment 3 and a 50 per cent response in Experiment 2 (Lots 15 and 9). Dosages ranging from 0.2 to 0.02 unit did not cause significantly different responses in Experiment 2 (Lots 8 and 11), whereas in Experiment 3 the responses to dosages from 0.1 to 0.01 unit were significantly different (Lots 15 and 17). The sample of reticulogen used in Experiment 2 was apparently of much lower growth-promoting potency than that used in Experiment 1 or 3.

Optimum growth responses were consistently obtained in all experiments with dosages of reticulogen at 0.4 and 0.5 units per bird per day. At lower dosages variations in potency could be noted. When higher levels were used the response was slightly less than maximal.

Four 15 U. S. P. unit liver concentrates were tested in Experiments 3 and 4 at a uniform dosage of 0.75 unit. This level allowed for a wide range in potency. The Armour liver extract and the Abbott liver extract were highly active, showing growth responses of 69 and 78 per cent respec-

TABLE I

*Average Weight and Comparative Response of Chicks to Various Supplements*

Experiment No.	Lot No.	Supplement	No. of chicks	Average weight, 4 wks.	Response
				<i>gm.</i>	<i>per cent</i>
1	1	None	12	147	
	2	3% condensed fish solubles	12	249	100
	3	Reticulogen (Lilly, 20 U. S. P. units per cc.) 0.4 unit per bird per day*	12	268	118
	4	Liver extract (Sharp and Dohme, 15 U. S. P. units per cc.) 0.4 unit per bird per day*	11	268	118
2	5	None	9	120	
	6	3% condensed fish solubles	11	220	100
	7	Reticulogen (Lilly, 20 U. S. P. units per cc.) 0.4 unit per bird per day*	10	198	78
	8	Reticulogen (Lilly, 20 U. S. P. units per cc.) 0.2 unit per bird per day*	9	173	53
	9	Reticulogen (Lilly, 20 U. S. P. units per cc.) 0.1 unit per bird per day*	9	170	50
	10	Reticulogen (Lilly, 20 U. S. P. units per cc.) 0.04 unit per bird per day*	10	174	54
3	11	Reticulogen (Lilly, 20 U. S. P. units per cc.) 0.02 unit per bird per day*	11	166	46
	12	None	12	159	
	13	3% condensed fish solubles	12	259	100
	14	Reticulogen (Lilly, 20 U. S. P. units per cc.) 1.0 unit per bird per day*	12	240	81
	15	Reticulogen (Lilly, 20 U. S. P. units per cc.) 0.1 unit per bird per day*	12	254	95
	16	Reticulogen (Lilly, 20 U. S. P. units per cc.) 0.02 unit per bird per day*	12	210	51
	17	Reticulogen (Lilly, 20 U. S. P. units per cc.) 0.01 unit per bird per day*	12	205	46
	18	Liver extract (Armour, 15 U. S. P. units per cc.) 0.75 unit per bird per day*	12	228	69
	19	Liver extract (Abbott, 15 U. S. P. units per cc., E-567) 0.75 unit per bird per day*	12	237	78
	20	Liver extract (Abbott, 15 U. S. P. units per cc., E-1043) 0.75 unit per bird per day*	12	169	10
4	21	Leucopterin, 500 $\gamma$ per 100 gm.	11	173	14
	22	None	12	198	
	23	3% condensed fish solubles	12	307	100
	24	Liver extract (Abbott, 15 U. S. P. units per cc., E-1125) 0.75 unit per bird per day*	11	170	-26

TABLE I—*Concluded*

Experiment No.	Lot No.	Supplement	No. of chicks	Average weight, 4 wks.	Response
				gm.	per cent
5†	25	None	12	188	
	26	3% condensed fish solubles	12	328	100
	27	Reticulogen (Lilly, 20 U. S. P. units per cc.) 0.4 unit per bird per day*	12	314	90
	28	Liver extract (Lilly, 20 U. S. P. units per cc., E-1108) 0.4 unit per bird per day*	12	289	72
	29	Liver concentrate (Lilly, anemia fraction, E-1180) 0.6 cc. per 100 gm. $\equiv$ 12% fresh liver	12	270	59
	30	Liver concentrate (Lilly, vitamin fraction, E-1181) 0.6 cc. per 100 gm. $\equiv$ 12% fresh liver	12	288	71
6†	31	None	11	184	
	32	3% condensed fish solubles	11	329	100
	33	95% ethanol extractives of liver (Abbott) 1.2 gm. per 100 gm. $\equiv$ 12% fresh liver	11	284	69
	34	3% dried brewers' yeast	9	194	7
	35	6% " " "	9	180	-3
	36	3% yeast extract	8	139	-31
7†	37	None	12	237	
	38	3% condensed fish solubles	11	330	100
	39	Reticulogen (Lilly, 20 U. S. P. units per cc.) 0.5 unit per bird per day*	12	329	99
	40	Liver extract (Abbott, 15 U. S. P. units per cc., E-1043) 0.5 unit per bird per day*	12	248	12
	41	0.5 unit reticulogen + 0.5 unit liver extract E-1043 per bird per day*	12	302	70
	42	Vitamin B <sub>12</sub> concentrate 1.5 $\gamma$ per 100 gm.	12	314	83
	43	Vitamin B <sub>12</sub> concentrate 3.0 $\gamma$ per 100 gm.	12	324	94
	44	<i>n</i> -Butanol-soluble fraction of condensed fish solubles fed at 0.032 gm. per 100 gm. ration $\equiv$ 6% condensed fish solubles	12	244	8
	45	<i>n</i> -Butanol-insoluble fraction of condensed fish solubles fed at 0.10 gm. per 100 gm. ration $\equiv$ 6% condensed fish solubles.	11	322	91

\* Injected intramuscularly.

† 0.03 per cent iodinated casein included in the basal ration.

tively (Lots 18 and 19). But two other preparations, E-1043 and E-1125, showed no growth-stimulating potency (Lots 20 and 24). All of these preparations had been tested clinically for pernicious anemia potency and found to be active.

Xanthopterin has given hematological responses in several experimental anemias (5-7). Jacobson and Good (8) were able to increase the potency of folic acid in the treatment of pernicious anemia patients by incubating folic acid with an enzyme capable of oxidizing xanthopterin to leucopterin. In order to test leucopterin for activity in the chick, this compound was fed directly in the basal ration at a level of 500  $\gamma$  per 100 gm. of diet. No significant growth response was observed (Lot 21). A sufficient amount of leucopterin was not available to permit tests at higher concentrations.

An experimental preparation (Lilly E-1108) more highly purified than reticulogen for antipernicious anemia potency was found to have slightly lower growth-stimulating potency than reticulogen (Lots 27 and 28). Preparation E-1108 contains 6 per cent of solids as compared with 10 per cent of solids in reticulogen. Although E-1108 caused a growth response of 72 per cent compared to 90 per cent for reticulogen, most of the growth activity was retained during the purification.

Several fractions obtained during the commercial production of liver extracts were tested. Two liver concentrates, E-1180 and E-1181, resulted from the separation of the preliminary crude extract into two fractions. One of these was further purified for use in anemia and the other was used as a source of vitamins. The anemia fraction, E-1180, contains about 10 times as much antipernicious anemia (APA) potency as the vitamin fraction E-1181. Both of these fractions were highly active in stimulating chick growth (Lots 29 and 30). The response with the vitamin concentrate was slightly higher than that of the anemia concentrate.

The APA factor is soluble in 70 per cent ethanol and quite insoluble in 95 per cent ethanol. A fraction containing the 95 per cent ethanol extractives of liver was fed in the basal ration at a level equivalent to 3 per cent liver powder. It was very active in stimulating chick growth (Lot 33). This fraction is normally discarded in the preparation of APA extracts. Cohn *et al.* (9) observed that APA-active material in liver was soluble in *n*-butanol. The substance occurring in condensed fish solubles which stimulates chick growth was clearly insoluble in *n*-butanol (Lot 45).

Betheil *et al.* (10) reported that liver is a better source of antithyrototoxic materials than yeast. Two samples of yeast were tested for growth-promoting potency in the chick. Dried brewers' yeast included in the basal ration at levels of 3 and 6 per cent did not cause any response (Lots 34 and 35). A water extract of yeast fed at 3 per cent resulted in some inhibition of growth (Lot 36).

To check the possibility that lack of any growth response with a clinically active liver extract might be due to toxic substances, further tests were made with liver extract E-1043. This preparation was inactive in promoting growth (Lot 40), while reticulogen containing the same number of APA units caused a growth response of 99 per cent (Lot 39). The combined administration of 0.5 unit of E-1043 along with 0.5 unit of reticulogen caused a growth response of 70 per cent (Lot 41).

Ott *et al.* (11) reported that crystalline vitamin B<sub>12</sub> has "animal protein factor" activity for the chick. They compared vitamin B<sub>12</sub> with liver powder and condensed fish solubles, all of which were highly active. To test the activity of vitamin B<sub>12</sub> under the conditions of our assay, a vitamin B<sub>12</sub> concentrate, supplied by Dr. D. F. Green, Merck and Company, Inc., Rahway, New Jersey, was mixed directly into the basal ration to provide 1.5  $\gamma$  and 3.0  $\gamma$  per 100 gm. of diet. The vitamin B<sub>12</sub> content of the concentrate had been determined microbiologically to be 2.0 mg. per pound. The response obtained was 83 per cent at 1.5  $\gamma$  per 100 gm. and 94 per cent at 3.0  $\gamma$  of vitamin B<sub>12</sub> per 100 gm. (Lots 42 and 43).

#### DISCUSSION

The data presented indicate that the stated U. S. P. potency of liver extracts does not serve as an accurate index of growth-promoting potency for the chick. It was observed that the response to reticulogen was much lower in Experiment 2 than in Experiment 1 or 3. Since the difference between the positive and negative controls was quite consistent, it is evident that some variation in potency occurs between different batches of the same commercial preparation.

Assuming a relationship between APA potency and growth-stimulating activity for the chick, the variation noted with one product could readily be accounted for by the inherent inaccuracy of the method used to establish APA units. Since the number of Addisonian pernicious anemia patients available for the standardization of commercial liver extracts are few and the individual responses variable, it is understandable that any statement of potency must necessarily represent an approximation. But even allowing for wide variation due to clinical standardization, it is unlikely that these differences could be great enough to cover the range in growth response from high activity to complete inactivity observed with these 15 U. S. P. unit liver extracts. Daniel *et al.* (12) found no relationship between the U. S. P. potency of liver extracts and their animal protein factor activity for *Lactobacillus casei*.

One of our inactive preparations, E-1043, was retested in combination with reticulogen to insure that the effect was not due to toxic substances. A normal growth response was obtained. This liver extract has been



found to be active in six patients for the remission of pernicious anemia in relapse.<sup>1</sup>

Several considerations should be kept in mind in dealing with this and other related evidence. First, growth is not a specific criterion. This is especially true when more than one unidentified growth stimulant is known to occur. Novak and Hauge (13) reported that a fat-soluble substance from distillers' dried solubles, referred to as vitamin B<sub>13</sub>, stimulated the growth of rats. Liver extract was also effective. The influence of this factor in growth assays for the animal protein factor in liver extracts would likely be small, due to the different solvent properties. However, it is highly probable that the series of vitamin B factors will be extended further.

Secondly, the remission of anemia is not a specific response. Pfiffner and Hogan (14) have presented an excellent review of the many substances which can act as hematopoietic agents. The similar physiological changes in the blood picture of several anemias, resulting from therapy with folic acid or with liver extracts containing little or no folic acid, have not been adequately explained.

Thirdly, even the highly refined liver extracts are still complex mixtures. Shive *et al.* reported that thymidine is present in high concentrations in liver extracts (15). The response of *Lactobacillus lactis* Dorner (LLD) has been related to APA potency and has been used to measure the vitamin B<sub>12</sub> content of liver extracts (16). Sauberlich and Baumann (17) have observed that liver extracts were very active in stimulating the growth of *Leuconostoc citrovorum*. Thymidine caused only a partial response. The concentration of the substance active for this organism did not correspond to LLD activity or to chick growth. Liver extract E-1043 was inactive for chick growth and was very low in LLD units. Yet this preparation was comparable to reticulogen for the growth stimulation of *Leuconostoc citrovorum*. A comparison of several liver extracts indicated that the growth response of chicks was related to the LLD activity.<sup>2</sup>

The amount of liver extract required to stimulate chick growth was very small. 0.01 unit of reticulogen contains approximately 50  $\gamma$  of solids; yet at this dosage (injected) the growth response was half maximal. Vitamin B<sub>12</sub> was effective when fed at a level of 1.5  $\gamma$  per 100 gm. However, the concentrate used did not exclude the presence of other factors such as those that occur in reticulogen. All of the extracts which stimulated chick growth were active for pernicious anemia therapy.

On the other hand, two 15 U. S. P. unit preparations mentioned previ-

<sup>1</sup> Data supplied by Dr. D. V. Frost, Abbott Laboratories, North Chicago, Illinois.

<sup>2</sup> Sauberlich, H. E., and Baumann, C. A., personal communication.

ously which are known to be active in the treatment of pernicious anemia were inactive in promoting chick growth. Also one crude fraction of liver containing 10-fold more APA potency than another fraction was less effective in stimulating growth. The 95 per cent ethanol extractives of liver which are discarded in the preparation of APA extracts were highly active for growth. The material from liver which is active for pernicious anemia has been reported to be soluble in *n*-butanol. The material from condensed fish solubles which stimulates chick growth is insoluble in *n*-butanol. Such evidence makes it difficult to rationalize the identity of the APA factor with that of the "animal protein factor;" however, slight differences in structure may account for these results.

It is likely that several compounds may be active in the human which show different responses in other species and in microorganisms. Equally important is the view that an interrelationship of several factors may be necessary to permit the synthesis and utilization of compounds whose total effect is measured by growth or hematological responses.

Stokstad *et al.* (18) used a chick assay to standardize the "animal protein factor" potency of microbially produced material which was active in the remission of pernicious anemia in two patients. Further studies are required to correlate the evidence presented here with the observed stimulation of growth in chicks fed cow manure (19) and chicken feces (20). The influence of the intestinal flora may have far reaching implications in the nutritional dysfunction expressed in pernicious anemia.

#### SUMMARY

A number of injectable liver extracts and crude liver fractions were tested for growth-stimulating potency in the chick. The stated U. S. P. potency was not related to the growth response. Reticulogen, at 0.01 unit per bird per day, caused a half maximal growth response. A vitamin B<sub>12</sub> concentrate effectively stimulated chick growth at a level of 1.5  $\gamma$  of vitamin B<sub>12</sub> per 100 gm. of ration.

Other liver extracts (15 U. S. P. units), active in the remission of pernicious anemia, were inactive for the growth of the chick. Two crude liver fractions which are discarded in the commercial preparation of APA extracts were highly active in promoting chick growth.

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## STUDIES ON THE CYCLOPHORASE SYSTEM

### V. THE OXIDATION OF PROLINE AND HYDROXYPROLINE\*

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(Received for publication, July 19, 1948)

The properties of the cyclophorase system, a complex of enzymes which catalyzes the complete oxidation of pyruvate and fatty acids by way of the tricarboxylic acid cycle, have been described in previous communications from this laboratory (1-4). Early in the course of these studies, it was observed that the oxidation of several natural amino acids likewise occurs in the cyclophorase system. Of the L-amino acids only glutamate, proline, and hydroxyproline are consistently oxidized at rapid and reproducible rates. Under favorable conditions, both glutamate and proline are oxidized completely to carbon dioxide, water, and ammonia, whereas hydroxyproline undergoes only partial oxidation. Unpublished observations<sup>1</sup> have shown that glutamate gains entrance into the tricarboxylic acid cycle by its oxidation to  $\alpha$ -ketoglutarate in a manner similar to that described for various other enzyme preparations of higher plant (5) and animal origin (6, 7). The present communication deals primarily with the oxidation of proline and hydroxyproline by pathways apparently hitherto undescribed.

#### Results

*Components of Proline Oxidizing System*—Maximal rates of oxygen consumption are observed during proline oxidation only when the following components are added to the thrice washed residue of rabbit kidney or liver homogenates: (1) adenosine-5-phosphate or adenosine triphosphate, (2) magnesium ions, (3) inorganic phosphate, and (4) an excess of substrate. The addition of coenzymes I or II, flavin-adenine dinucleotide, or cytochrome *c* is without effect on the rate of oxygen uptake in the complete system. A moderate, but consistent, reduction of oxygen consumption is observed in the absence of either adenylic acid or magnesium ions (*cf.* Fig. 1). When both of these components are lacking, the oxygen uptake is one-quarter, or less, of that observed with the complete system. Both the

\* This investigation was supported by grants from the Rockefeller Foundation, the Williams-Waterman Fund of the Research Corporation, and the American Cancer Society.

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<sup>1</sup> Loomis, W. F., unpublished observations.

magnesium and adenylic acid requirements become increasingly apparent with time during the course of an experiment.

The rate of proline disappearance is, however, essentially the same in either the complete or partially reconstituted system. Table I contains the data relating the disappearance of proline and oxygen uptake in several representative experiments. It may be noted that approximately 10 atoms of oxygen are consumed for each molecule of proline disappearing in the

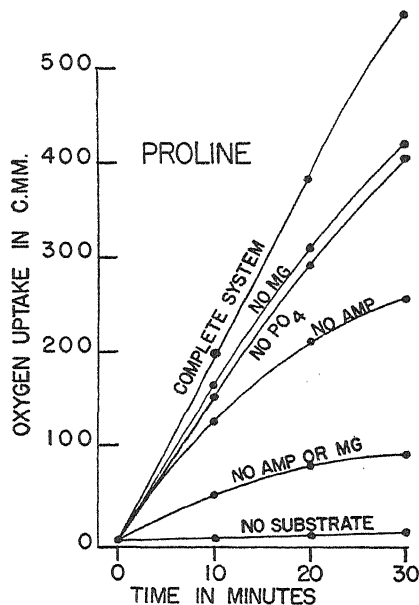
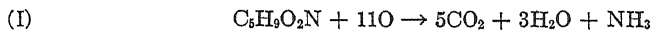


FIG. 1. Oxidation of L-proline in the cyclophorase system. Each manometer vessel contained 1.0 ml. of the third residue of rabbit kidney homogenate, 0.3 ml. of 0.04 M phosphate buffer of pH 7.3, 0.3 ml. of 0.01 M adenosine-5-phosphate, 0.2 ml. of 0.04 M magnesium sulfate, 0.3 ml. of 0.1 M L-proline, water to a final volume of 2.8 ml., 0.2 ml. of 6 N NaOH in the center well, and oxygen in the gas space, except for the omissions indicated. The vessels were equilibrated at 38° for 5 minutes before the taps were closed.

complete system. This ratio approaches closely the value for the complete oxidation of proline (Reaction I).



With the incomplete system (no adenylic acid or magnesium added), only slightly more than 2 atoms of oxygen are consumed for each molecule of proline which disappears.

*Products of Oxidation*—The liberation of ammonia and the accumulation

of bisulfite-binding products were estimated in both the complete and incomplete systems in an attempt to clarify the nature of the initial oxidation reactions (*cf.* Table II). The oxidation of proline in the complete system

TABLE I  
*Oxidation of Proline in Cyclophorase System*

System	Time	Oxygen consumed	Disappearance of proline	$\frac{\Delta \text{oxygen}}{\Delta \text{proline}}$
	<i>min.</i>	<i>microatoms</i>	<i>micromoles</i>	
Complete	40	50	5.5	9.1
"	100	116	12.0	9.7
"	130	158	14.8	10.6
No ATP or Mg	55	18	8.2	2.2
" " " "	100	25	10.0	2.5

The complete system consisted of 1 ml. of the third residue of rabbit kidney, 0.3 ml. of 0.04 M phosphate buffer of pH 7.3, 0.3 ml. of 0.01 M adenosine triphosphate (ATP), 0.2 ml. of 0.04 M magnesium sulfate, 0.3 ml. of 0.1 M L-proline, water to a final volume of 2.8 ml., alkali in the center well, and oxygen in the gas space. Temperature 38°. All of the components were introduced into the main compartment of the manometer vessels and equilibrated for 5 minutes before closing the taps. Oxygen uptake was determined as described in the experimental section.

TABLE II  
*Liberation of Ammonia and Accumulation of Bisulfite-Binding Products during Oxidation of Proline and Hydroxyproline*

Substrate	System	Oxygen uptake	Substrate disappearing	Bisulfite binding	Ammonia liberated
		<i>microatoms</i>	<i>micromoles</i>	<i>micromoles</i>	<i>micromoles</i>
Proline	Complete	125	13.8	0	15.0
"	No ATP or Mg	20.3	10.4	4.3	0
Hydroxyproline	Complete	23.3*	7.5	3.2	3.7
"	No ATP or Mg	7.6	7.7	6.0	0.7

The contents of the manometer vessels were as described in Table I. Each vessel was set up in triplicate and the contents of the three pooled at the end of the experiment. One aliquot was precipitated with 5 per cent trichloroacetic acid for the bisulfite estimation, while a second aliquot was precipitated with sodium tungstate and H<sub>2</sub>SO<sub>4</sub> for the amino acid and ammonia estimations. The total time at 38° was 30 to 35 minutes.

\* The theoretical oxygen uptake for the complete oxidation of 7.5 micromoles of hydroxyproline is 75 microatoms.

yields an amount of ammonia which corresponds to the proline consumed, the oxygen uptake approaches that required for complete oxidation, and no bisulfite-binding product accumulates. On the other hand, little or no

ammonia is liberated in the incomplete system and approximately half of the proline can be accounted for in the form of a carbonyl-containing product. When hydroxyproline is the added substrate in the complete system, the oxygen uptake falls far short of the amount required for complete oxidation. In the incomplete system, the oxygen consumption is limited to 1 atom per molecule of substrate, and the hydroxyproline is almost quantitatively converted to a bisulfite-binding product.

Since the studies of Krebs (8, 9), Blanchard *et al.* (10), and Stumpf and Green (11) have established  $\alpha$ -keto- $\delta$ -aminovalerate as the oxidation product of proline, this compound was prepared from DL-proline by means of the flavoprotein D-amino acid oxidase extracted from an acetone powder of sheep kidney. An ultrafiltrate of the final reaction mixture, which contained both L-proline and  $\alpha$ -keto- $\delta$ -aminovalerate, was then tested in the

TABLE III  
*Test for  $\alpha$ -Keto- $\delta$ -aminovalerate Oxidation*

Substrates added	System	Oxygen uptake	Final bisulfite binding
		<i>microatoms</i>	<i>micromoles</i>
Proline alone, 50 micromoles	Complete	139	0.9
" " 50 "	No ATP or Mg	13	8.6
" 50 micromoles, + $\alpha$ -keto- $\delta$ -aminovalerate, 17.7 micromoles	Complete	121	18.9
" "	No ATP or Mg	16	23.7

The contents of the manometer vessels were as described in Table I, except for the indicated substrates. The  $\alpha$ -keto- $\delta$ -aminovalerate was contained in 0.5 ml. of an ultrafiltrate prepared as described in the experimental section. Total time in water bath at 38°, 100 minutes.

cyclophorase system (Table III). Under conditions which were optimal for the oxidation of proline, there was no disappearance of the  $\alpha$ -keto- $\delta$ -aminovalerate. The final bisulfite binding obtained in the complete and incomplete systems corresponded to the amount of ketovalerate added plus the bisulfite-binding product contributed by proline oxidation.

The 2,4-dinitrophenylhydrazone of the  $\alpha$ -keto- $\delta$ -aminovaleric acid exhibited properties similar to those previously described (9-11). The slender orange-yellow crystals, which were readily recrystallized from hot 2 N HCl, melted with decomposition at 222-224°. The corresponding derivative of the product obtained from incomplete proline oxidation in the cyclophorase system differed in several respects. A fine, dark reddish brown, amorphous precipitate formed slowly over a 10 day period at room temperature. The solubility of the hydrochloride in hot 2 N HCl was too limited to permit recrystallization. Attempts to achieve recrystallization



from various organic solvents were likewise unsuccessful because of solubility limits. Consequently, the precipitate was washed several times with warm dilute HCl and water and was dried *in vacuo* over  $P_2O_5$  and NaOH. The dried material melted with decomposition at 211–214°. After admixture with the hydrazone of  $\alpha$ -keto- $\delta$ -aminovalerate, the melting point was 195°. Elementary analysis yielded values which were reasonably consistent with those calculated for the 2,4-dinitrophenylhydrazone hydrochloride of either  $\alpha$ -keto- $\delta$ -aminovaleric acid or the semialdehyde of glutamic acid.

*Analysis*— $C_{17}H_{15}N_5O_6 \cdot HCl$ . Calculated. C 37.99, H 4.06, N 20.14  
Found. “ 37.83, “ 3.91, “ 19.40

The results obtained with hydroxyproline appear to be more revealing. The product which accumulates during the incomplete oxidation of hydroxyproline forms a 2,4-dinitrophenylhydrazine derivative sparingly soluble in 2 N HCl. An amorphous precipitate, orange to brick-red in color, was collected over a 12 day period at room temperature. As in the case of the proline derivative, attempts to achieve recrystallization from HCl and various organic solvents were unsuccessful. The washed and dried precipitate melted with decomposition at 151–154°. The material dissolved readily in dilute NaOH to yield an intensely purple solution with an absorption maximum between 550 and 560 m $\mu$ , a characteristic property of many 2,4-dinitrophenylosazones. The analytical data are of particular interest in that they correspond to the calculated values for the 2,4-dinitrophenylosazone of  $\gamma$ -hydroxyglutamic semialdehyde rather than the hydrazone of  $\alpha$ -keto- $\gamma$ -hydroxy- $\delta$ -aminovaleric acid.

*Analysis*— $C_{17}H_{15}N_5O_{10} \cdot HCl$ . Calculated. C 37.68, H 2.97, N 23.27  
Found. “ 39.37, “ 2.88, “ 23.27

In view of these findings, it seemed quite likely that the initial oxidation product of proline in the cyclophorase system was glutamic semialdehyde. An ultrafiltrate of the final reaction mixture of proline in the incomplete system was concentrated by lyophilization and tested with the liver aldehyde oxidase of Gordon *et al.* (12) and with the xanthine oxidase of Corran and Green (13). Either of these enzymes might reasonably have been expected to reveal the presence of an aldehyde in the reaction mixture. However, in both cases, the intermediate derived from incomplete proline oxidation proved to be inert as a substrate.

*Formation of Glutamic Acid*—Glutamic acid is an intermediate of proline oxidation in the cyclophorase system of enzymes. Its presence as an end-product in the incomplete system was demonstrated by means of the specific glutamic acid decarboxylase of dried *Escherichia coli* (14). In addition, the chloramine-T method (15) yielded an equivalent amount of

succinic acid, which was identified both by the succinoxidase of pig heart and by its melting point, 183°. With the recognition that glutamic acid was the second product of proline oxidation, it became possible to perform balanced operations in the complete and incomplete systems, as shown in Table IV. The yield of glutamic acid in the incomplete system accounts for approximately one-half of the proline oxidized. The remainder is accounted for as the bisulfite-binding intermediate and by ammonia liberation. In the complete system, neither of the intermediates accumulates in any significant quantity and essentially all of the proline undergoes deamination.

TABLE IV  
*Balance Sheet of Proline Oxidation*

	Incomplete system (no ATP or Mg)	Complete system
	<i>microatoms</i>	<i>microatoms</i>
1. Oxygen consumed.....	131	435
	<i>micromoles</i>	<i>micromoles</i>
2. Proline disappearing.....	65.1	57.5
3. Bisulfite binding.....	22.5	1.0
4. Glutamic acid formed.....	25.5	5.3
5. Ammonia liberated.....	15.0	48.5
Total, (3) - (5).....	63.0	54.8

The complete system contained 6.0 ml. of the third residue of kidney, 1.8 ml. of phosphate buffer of pH 7.3, 1.8 ml. of 0.01 M ATP, 1.2 ml. of 0.04 M  $\text{MgSO}_4$ , 1.8 ml. of 0.1 M proline, water to a final volume of 16.8 ml., and oxygen in the gas space. The mixture was equally divided in six manometer vessels and pooled at the end of the experiment. Total time in bath at 38°, 60 minutes.

*Specificity of Amino Acid Oxidases*—As mentioned previously, the only amino acids of the L configuration oxidized under the conditions described are proline, hydroxyproline, and glutamate.<sup>2</sup> The D isomers of these three amino acids are inactive as substrates. When both proline and hydroxyproline are added to the system in excess, the observed rate of oxygen uptake approximates that of proline alone (*cf.* Table V). There is, however, a partial summation of the individual rates when proline and glutamate are oxidized simultaneously. Thus, it appears likely that the same enzyme catalyzes the oxidation of proline and hydroxyproline, but

<sup>2</sup> Subsequent experiments by Dr. J. Still of this laboratory have shown that L-alanine is oxidized in this system in the presence of 0.016 M  $\text{NaHCO}_3$  or catalytic amounts of one of the members of the citric acid cycle. Cyclophorase enzyme preparations will catalyze the oxidation of D-aspartic acid but not of the L isomer.

not that of glutamate. The glutamic acid oxidase is further differentiated from the proline enzyme by its adenylic acid and magnesium requirements.

Of the various heterocyclic compounds tested in the cyclophorase system, only  $\alpha$ -picolinic acid was oxidized. Pyrrolidine, pyrrolidonecarboxylic acid, pyrroline, pyrrole,  $\alpha$ -picoline, nicotinic acid, tetrahydrofurfural, and tetrahydrofurfuryl alcohol were found to be inactive.

*General Properties of Proline Oxidase*—Both rabbit kidney and liver provide rich sources of the proline-oxidizing enzyme. The freshly prepared enzyme has a  $QO_2$  (c.mm. of oxygen per 60 minutes per mg., dry weight) of approximately 25 for proline and 15 for hydroxyproline. A loss of activity, amounting to about 50 per cent per 24 hours, occurs on storage of the enzyme at 4°. On the other hand, almost complete inactivation occurs when the enzyme is incubated at 38° for 15 minutes in the absence of substrate, a

TABLE V  
*Oxidation of Pairs of Amino Acids*

Substrate	Oxygen uptake
	<i>c.mm.</i>
Proline.....	261
Hydroxyproline.....	118
Glutamate.....	385
Proline + hydroxyproline.....	271
“ + glutamate.....	454

The additions to the manometer vessels were made as described in Table I, except that 50 micromoles of the indicated substrates were used. The oxygen uptake is that recorded during the first 20 minutes at 38°.

property shared by the majority of the enzymes of the cyclophorase complex. The enzyme is also inactivated below pH 6.0 during the homogenization, by exposure to organic solvents greater than 10 per cent by volume, and by freezing or drying.

*Inhibitors*—Arsenite is an effective inhibitor of the various cyclophorase enzymes which exhibit adenylic acid and magnesium requirements. Essentially complete inhibition of glutamate,  $\alpha$ -ketoglutarate, or malate oxidation occurs in the presence of 0.005 M arsenite. With proline as the added substrate, the same concentration of arsenite depresses the oxygen consumption to approximately the same level as that observed with the incomplete system. Furthermore, the addition of arsenite to the incomplete system has no appreciable effect on either the oxygen consumption or the formation of the bisulfite-binding product (*cf.* Table VI). Consequently, it may be concluded that arsenite exerts little or no inhibitory

effect on the reactions concerned with the direct oxidation of proline to glutamate.

Both 0.001 M cyanide and traces of capryl alcohol are potent inhibitors of proline, hydroxyproline, and glutamate oxidation (Table VII). In this respect, the cyclophorase enzymes are similar to the L-amino acid oxidase observed in rat kidney slices by Krebs (8) and that described in *Proteus*

TABLE VI  
*Arsenite Inhibition of Proline Oxidation*

System	Arsenite	Oxygen uptake	Final bisulfite binding
	<i>M</i>	<i>microatoms</i>	<i>micromoles</i>
Complete .....		48.3	0.3
" .....	0.005	11.6	5.7
No ATP or Mg .....		9.0	4.5
" " " " .....	0.005	10.6	6.2

Complete system as described in Table I. Duration of experiment at 38°, 90 minutes.

TABLE VII  
*Inhibition of Amino Acid Oxidation by Cyanide and Capryl Alcohol*

Substrate	Inhibitor	Oxygen uptake	Inhibition
		<i>c.mm.</i>	<i>per cent</i>
Glutamate		425	
"	Cyanide	54	87
"	Capryl alcohol	13	97
Proline		308	
"	Cyanide	56	82
"	Capryl alcohol	9	97
Hydroxyproline		163	
"	Cyanide	67	59
"	Capryl alcohol	7	96

The additions to the manometer vessels were made as described in Table I. The final concentration of cyanide was 0.001 M. 0.015 ml. of capryl alcohol was added to a final volume of 2.8 ml., but this amount exceeds its solubility limit. The figures for oxygen uptake are those recorded during the first 20 minutes at 38°.

*vulgaris* (11). Neither of these agents inhibits either D-amino acid oxidase (8) or the L-amino acid oxidase isolated from rat kidney and liver (10).

#### EXPERIMENTAL

*Preparation of Enzymes*—The cyclophorase enzyme was prepared as described in a previous communication from this laboratory (1). Fresh,

chilled rabbit kidneys were homogenized with 10 volumes of ice-cold 0.9 per cent KCl in a Waring blender,  $N$  NaOH being added dropwise during the homogenization to maintain the pH between 7.0 and 7.5. After centrifugation in the cold, the residue was washed two additional times with the original volume of KCl and stored at 4°.

The liver aldehyde oxidase was prepared by the method of Gordon *et al.* (12) and the xanthine oxidase of milk by the method of Corran and Green (13). Dr. I. C. Gunsalus generously supplied us with the sample of dried *Escherichia coli* (strain 86-M) which contained the glutamic acid decarboxylase used in the identification experiments. This preparation was free of the lysine and arginine decarboxylases. Glutamic acid estimations were performed by the usual manometric technique with 10 mg. of the *Escherichia coli* preparation suspended in 2.0 ml. of 0.075  $M$  phthalate buffer of pH 5.0 in the main compartment, 1.0 ml. of sample tipped in from the side arm after equilibration at 38°, and 5 per cent  $CO_2$  in nitrogen in the gas space. Recoveries with known amounts of glutamic acid averaged 85 per cent.

**Manometry**—The usual manometric procedures were employed except that the substrate was mixed with the cyclophorase enzyme at the start of the experiment in order to avoid the inactivation which results from equilibrating the enzyme at 38° in the absence of substrate. In those experiments in which the oxygen consumption was related to other measurements, the total oxygen uptake was estimated by including an extrapolation correction for the 5 minute equilibration period.

**Preparation of  $\alpha$ -Keto- $\delta$ -aminovalerate**—D-Amino acid oxidase was extracted in water from an acetone powder of sheep kidney according to the method of Krebs (9). The following were added to a large reaction vessel: 25 ml. of the extract adjusted to pH 8.3 with  $Na_2CO_3$ , 600 mg. of DL-proline, and oxygen in the gas space. After shaking for 80 minutes at 38°, the reaction mixture was deproteinized by ultrafiltration through a colloid membrane in the apparatus devised by Simms and Stillman (16). The ultrafiltrate, which contained 35 micromoles of bisulfite-binding material per ml., was then used for tests with the cyclophorase system and for the preparation of the 2,4-dinitrophenylhydrazone.

**Preparation of 2,4-Dinitrophenylhydrazine Derivatives**—A mixture containing 50 ml. of the third residue of rabbit kidney, 18 ml. of 0.04  $M$  phosphate buffer of pH 7.3, 16 ml. of 0.1  $M$  proline or hydroxyproline, and 40 ml. of water was placed in a large reaction vessel with oxygen in the gas space and shaken for 3 hours at 38°. The final reaction mixture was deproteinized by ultrafiltration and concentrated to 5 ml. by lyophilization at 0°. The concentrated filtrate was then mixed with 45 ml. of a saturated solution of 2,4-dinitrophenylhydrazine in 2.2  $N$  HCl, filtered, and

held at room temperature for 10 to 12 days. The precipitate was washed several times with warm  $N$  HCl and water and dried *in vacuo* over  $P_2O_5$  and NaOH. The yield of the hydroxyproline derivative was 131 mg., approximately 67 per cent of theory on the basis of the initial bisulfite binding. Elementary analyses were performed by Dr. W. Saschek of the Department of Biochemistry.

*Analytical Methods*—Proline and hydroxyproline were estimated by a modification of the method of Folin (17). Tungstate filtrates (1:10) were prepared of the initial and final reaction mixtures. An aliquot containing approximately 2 micromoles of the amino acid was made alkaline by the addition of 0.5 volume of 6  $N$  NaOH and was freed of ammonia by passing a brisk stream of steam through the solution for 5 minutes. The pH was then readjusted to 9.0, the volume brought to 5 ml., and 1 ml. of 0.5 per cent  $\beta$ -naphthoquinone sulfonate added. After 18 hours in the dark, the following reagents were added: 1 ml. of 25 per cent acetic acid in 2.5 per cent sodium acetate, 1 ml. of 4 per cent sodium thiosulfate, and water to a final volume of 15 ml. The optical density was measured at 525  $m\mu$  in the Coleman junior spectrophotometer. Since the accumulation of glutamic semialdehyde and glutamic acid in the incomplete system interferes with the estimation of proline disappearance, it was necessary to introduce appropriate corrections for these substances. Ammonia was estimated by micro-Kjeldahl distillation, bisulfite binding by the method of Clift and Cook (18), and the routine glutamic acid estimations by the chloramine-T method (15).

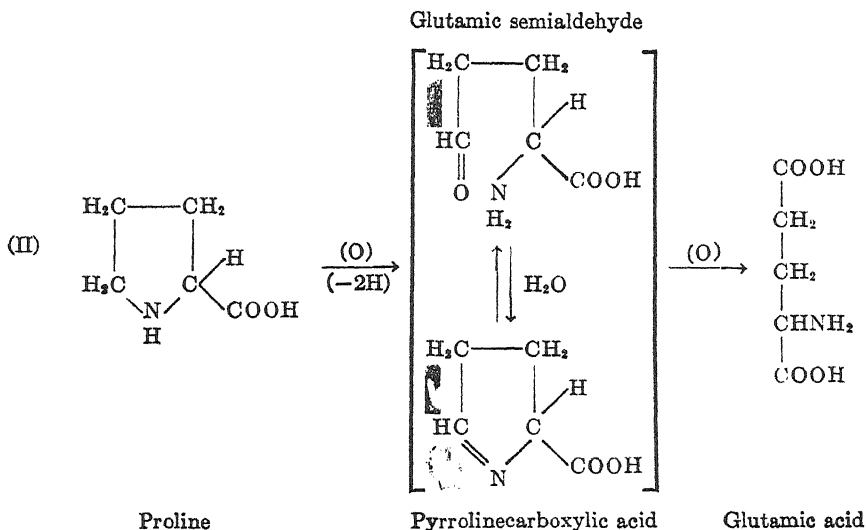
*Sources of Special Chemicals*—L-Proline was obtained from the H. M. Chemical Company, Ltd., DL-proline from Hoffmann-La Roche, and hydroxy-L-proline from Eastman. We are indebted to Dr. H. Waelsch for a sample of pyrrolidonecarboxylic acid. Adenosine triphosphate was prepared from rabbit skeletal muscle (19); adenosine-5-phosphate was supplied by the Schwarz Laboratories.

#### DISCUSSION

The conversion of proline to glutamic acid in the animal body has been established by a variety of experimental techniques (20–22). Of particular interest is the finding of Stetten and Schoenheimer (23) that the organ proteins of rats fed proline labeled with  $N^{15}$  and deuterium yield glutamic acid containing appreciable amounts of both isotopes. Thus, it is apparent that both the nitrogen and the carbon skeleton of glutamic acid are derived in part from proline. The nature of the intermediates, however, has remained a subject of considerable interest and speculation.

The demonstration that  $\alpha$ -keto- $\delta$ -aminovaleric acid is the oxidation product of proline with both the D- (9) and L-amino acid oxidases of animal

tissues (10) has implicated this compound as a possible intermediate in the conversion of proline to glutamic acid. On this basis, Krebs (9) suggested the following as a possible metabolic pathway: (a) the oxidative opening of the pyrrolidine ring between the N and  $\alpha$ -carbon to yield  $\alpha$ -keto- $\delta$ -aminovaleric acid, (b) deamination to the semialdehyde of  $\alpha$ -ketoglutarate, (c) oxidation to  $\alpha$ -ketoglutarate, and (d) the formation of glutamic acid by transamination. This sequence of reactions necessarily implies an exchange of the amino acid N with that in the metabolic pool of the body. An alternative scheme, by which the proline N is retained in glutamic acid, has been proposed by Stetten and Schoenheimer (23); *viz.*, the dehydrogenation of proline to pyrrolinecarboxylic acid, hydrolysis of the ring



between the N and  $\delta$ -carbon, and oxidation to glutamic acid. The possibility that either pyrrolidonecarboxylic acid (20-22) or  $\alpha$ -amino- $\delta$ -hydroxyvaleric acid (21) is an intermediate in this process appears to have been satisfactorily excluded.

The results obtained with the cyclophorase enzymes indicate that the conversion of L-proline to glutamic acid in this system occurs by way of the reactions proposed by Stetten and Schoenheimer (Reaction II).

Pyrrolinecarboxylic acid is presumed to be the intermediate present within the physiological range of pH. The hydrolytic cleavage of the pyrroline may occur only during its subsequent oxidation to glutamic acid or in the acid solutions employed in bisulfite binding or the formation of hydrazine derivatives. This would account for our failure to demonstrate

glutamic semialdehyde in the reaction mixture by means of the liver aldehyde oxidase or xanthine oxidase.

In the absence of added adenylic acid and magnesium ions, the oxidation of proline proceeds only as far as glutamic acid. The addition of these components to the system restores the oxidation of glutamate to  $\alpha$ -ketoglutarate and, hence, permits the complete oxidation of proline to proceed via the tricarboxylic acid cycle. There is no reason for believing that the glutamic acid formed during proline oxidation arises through transamination, since neither alanine nor aspartic acid is present in the system in appreciable amounts.

Although hydroxyproline is apparently oxidized to the corresponding pyrrolinecarboxylic acid, the subsequent oxidation reactions which it undergoes have yet to be identified. By analogy with proline,  $\gamma$ -hydroxyglutamic acid may be suggested as a possible intermediate in the oxidation of hydroxyproline. In any event, the complete oxidation of hydroxyproline has not been observed in this enzyme preparation.

The proline oxidase of the cyclophorase system is not identical with the flavoprotein L-amino acid oxidase isolated from rat kidney and liver (10). The latter enzyme withstands freezing, drying with acetone, a pH as low as 4.5, and a temperature of 60° for 5 minutes in the absence of substrate. One of the enzymes is readily extracted from tissues, while the other is associated with the particulate material of the cell. Finally, the two enzymes differ in their specificities, the products which they yield, and their sensitivity to such inhibitors as capryl alcohol and cyanide.

#### SUMMARY

The cyclophorase system of enzymes catalyzes the complete oxidation of L-proline to carbon dioxide, water, and ammonia by way of glutamic acid and the tricarboxylic acid cycle. The proline oxidase of this system is not identical with any of the amino acid oxidases previously described.

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## STUDIES ON THE CYCLOPHORASE SYSTEM

### VI. THE COUPLING OF OXIDATION AND PHOSPHORYLATION\*

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(Received for publication, July 19, 1948)

In Papers I to V of this series (1-5), there were presented the details of the chemical reactions which are involved in the complete oxidation of pyruvic acid, fatty acids, and certain amino acids by the cyclophorase-containing enzyme complex of rabbit kidney and liver. The present communication deals with the phosphate transformations which accompany these reactions and with some of the mechanisms involved.

#### Results

*Formation of Inorganic Pyrophosphate*—When the kidney or liver enzyme at the third residue stage ( $R_3K$  or  $R_3L$ )<sup>1</sup> catalyzes the oxidation of succinate in the presence of the usual components (cf. Table I), a disappearance of orthophosphate is observed. Fluoride in 0.03 M concentration significantly augments this effect. The amount of orthophosphate which disappears is small and varies somewhat from one enzyme preparation to another. In addition, time curves show that the amount removed within the first few minutes of the experiment is approximately half of that observed at the end of 1 hour, although oxygen consumption continues at its initial rate. It thus appears likely that two competing processes operate simultaneously, one involving the disappearance and the other the regeneration of orthophosphate. Phosphate esterification coincident with the oxidation of various members of the citric acid cycle and glutamate is shown in Table II.

The disappearance of orthophosphate is accompanied by the formation

\* This investigation was aided by grants from the Williams-Waterman Fund of the Research Corporation, the Rockefeller Foundation, the American Cancer Society, and Eli Lilly and Company. The studies dealing with the specificity of dinitrophenol derivatives and analogues were carried out under a contract with the Office of the Quartermaster Corps.

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<sup>1</sup>  $R_3K$  and  $R_3L$  designate the triply washed residues of kidney and liver homogenates respectively. Homogenization and washing in 0.9 per cent KCl solutions were performed as described in Paper I of this series (1).

of a phosphate ester which is readily hydrolyzed by  $N$  HCl at  $100^\circ$ . This compound was isolated as a barium salt by the procedure described in the experimental section. A sample of reagent grade sodium pyrophosphate

TABLE I  
*Components Required for Pyrophosphate Formation*

	Oxygen uptake	Disappearance of inorganic phosphate	Acid-labile phosphate	Pyrophospha- tase-labile phosphate
	<i>microatoms</i>	<i>micromoles</i>	<i>micromoles</i>	<i>micromoles</i>
Complete system. . . . .	94.0	9.6	6.8	6.8
No substrate. . . . .	5.8		0.8	0.4
" adenosine-5-phosphate. . . . .	80.4	7.3	4.6	4.8
" magnesium. . . . .	87.2	4.7	3.3	2.9
" fluoride. . . . .	94.7	-0.2	0.5	0.6

The complete system consisted of 1 ml. of  $R_3L$ , 0.2 ml. of 0.1  $M$  phosphate buffer of pH 7.3, 0.3 ml. of 0.01  $M$  adenosine-5-phosphate, 0.2 ml. of 0.1  $M$  magnesium chloride, 0.3 ml. of 0.33  $M$  sodium fluoride, 0.3 ml. of 0.5  $M$  succinate, and water to a final volume of 2.8 ml.; alkali in the center well and oxygen in the gas space. The other vessels were the same except for omission of the indicated component. The total time in the bath at  $38^\circ$  was 50 minutes.

TABLE II  
*Pyrophosphate Formation during Oxidation of Different Substrates*

Substrate	Oxygen uptake	Disappearance of inorganic phosphate	Acid-labile phosphate
	<i>microatoms</i>	<i>micromoles</i>	<i>micromoles</i>
None. . . . .	0		0.1
$\alpha$ -Ketoglutarate (30 micromoles) . . . .	10.0	7.6	6.6
Succinate (100 micromoles) . . . . .	25.9	7.2	6.6
Malate (30 micromoles) . . . . .	8.4	4.4	4.5
Citrate (30 " ) . . . . .	7.3	7.7	6.8
Glutamate (30 micromoles) . . . . .	8.3	5.5	5.5

Each manometer vessel contained 1 ml. of  $R_3L$ , 0.2 ml. of 0.1  $M$  phosphate buffer of pH 7.3, 0.3 ml. of 0.01  $M$  adenosine-5-phosphate, 0.2 ml. of 0.1  $M$  magnesium chloride, 0.2 ml. of 0.67  $M$  sodium fluoride, the indicated amount of substrate, and water to a final volume of 2.8 ml.; oxygen in the gas space. The vessels were iced before addition of the enzyme. The oxygen uptake was corrected to include a 5 minute equilibration period. The total time in the bath at  $38^\circ$  was 30 minutes.

was carried through the isolation procedure simultaneously for the purpose of comparison. The analytical data for the two samples are in fairly good agreement (*cf.* Table III), but it should be noted that the analyses of salts of pyrophosphoric acid are not entirely reliable, owing both to the difficul-

ties inherent in drying the samples satisfactorily and to the instability of pyrophosphoric acid in acid solutions (*cf.* Mann (6) for a comprehensive study of the isolation and analysis of inorganic pyrophosphates).

The pyrophosphate content of the isolated material was also estimated by means of a pyrophosphatase of yeast (7) which does not hydrolyze either metaphosphate, hexose phosphate, or adenosine triphosphate. A solution containing 2.4 mg. of the sample per ml. should have liberated 10.7 micromoles of orthophosphate after treatment with the yeast pyrophosphatase, assuming the isolated material to be pure barium pyrophosphate. Enzymatic hydrolysis actually liberated 9.1 micromoles of orthophosphate, indicating a purity of approximately 85 per cent. Orthophosphate present in the solution prior to enzymatic hydrolysis (0.87 micromole) accounted for slightly more than half of the impurity. The pyrophosphate was further

TABLE III  
*Analyses of Barium Pyrophosphate Samples*

	Unknown	Known
	<i>per cent</i>	<i>per cent</i>
Inorganic P.....	1.15	0.86
Acid-hydrolyzable P.....	10.8	11.4
Total P.....	13.4	12.8
(Inorganic P)/(total P).....	8.6	6.7
(Acid-hydrolyzable P)/(total P).....	80.5	89.0
Pentose.....	0.9	

Samples of 5 to 10 mg. of the barium salts were dissolved in dilute HCl just before analysis. Acid hydrolysis was performed in *N* HCl at 100° for 7 minutes. The theoretical total P for Ba<sub>2</sub>P<sub>2</sub>O<sub>7</sub> is 13.8 per cent. We are indebted to Dr. Sherman R. Dickman of this laboratory for carrying out the isolation and analytical procedures.

distinguished from metaphosphate by its inability to precipitate egg albumin.

The possibility remained that inorganic pyrophosphate was not present as such in the enzyme mixture, but was formed from some organic ester during the isolation procedure. However, analysis of the original trichloroacetic acid filtrate, prior to any purification procedure, has shown that essentially all of the acid-labile phosphate was estimated as inorganic pyrophosphate with the yeast pyrophosphatase (*cf.* Table I, last column). Under optimal conditions, such as a short incubation period and high fluoride concentration, the amount of pyrophosphate P formed never exceeded 0.9 microatom per microatom of oxygen consumed.

Cori and Ochoa (8) have mentioned unpublished experiments in which they succeeded in isolating crystalline sodium pyrophosphate from di-

alyzed dispersions of rat liver following the aerobic oxidation of glutamate, pyruvate, or succinate. It appears likely that the conditions of their experiments were very similar to those reported here.

*Use of Phosphate Acceptor Systems*—The addition of glucose to the first residue of the kidney homogenate ( $R_1K$ ) greatly increased the amount of inorganic phosphate which disappeared coincident with oxidation. Glucose had a much less striking effect when added to the third residue ( $R_3K$ ), indicating that an enzyme essential for the phosphorylation of glucose was removed during the standard preparation of the cyclophorase system. The supernatant of the first residue, when added to the  $R_3K$ , restored the capacity to phosphorylate glucose. The effect of the supernatant was

TABLE IV  
*Components Required for Aerobic Phosphorylation of Hexose*

	Oxygen uptake	Disappearance of inorganic phosphate	P:O
	<i>microatoms</i>	<i>micromoles</i>	
No substrate.....	0		
Complete system.....	13.4	35.6	2.66
No adenosine-5-phosphate.....	5.5	5.7	1.04
“ magnesium.....	9.7	10.3	1.06
“ fluoride.....	25.1	34.5	1.37
“ hexokinase.....	14.7	10.0	0.68
“ fructose.....	13.6	3.7	0.27

The complete system consisted of 1 ml. of  $R_3K$ , 0.5 ml. of 0.1 M phosphate buffer of pH 7.3, 0.3 ml. of 0.01 M adenosine-5-phosphate, 0.2 ml. of 0.1 M magnesium chloride, 0.1 ml. of 1.0 M fructose, 0.1 ml. of 0.33 M sodium fluoride, 0.02 ml. of yeast hexokinase, 0.3 ml. of 0.1 M  $\alpha$ -ketoglutarate, and water to a volume of 2.8 ml.; alkali in the center well and oxygen in the gas space. The other manometer vessels were identical except for omission of the indicated component. Extrapolation type of experiment (see the text). Total time, 23 minutes at 25°.

reproduced by a yeast hexokinase prepared by the method of Berger *et al.* (9) or a muscle hexokinase prepared by the method of Cori and Slein (10). Since hexokinases catalyze the transfer of phosphate from adenosine polyphosphate to hexose or hexose phosphate, it was inferred that the adenylic system serves as the catalytic phosphate carrier between the cyclophorase and hexokinase-glucose systems.

The data in Table IV show that magnesium ions, adenylic acid, hexokinase, hexose, and substrate are essential components for the esterification of phosphate. The optimal concentrations of the various components are 0.003 to 0.03 M for magnesium ions, 0.01 M or greater for adenylic acid, and an excess of hexose and substrate. The maximal hexokinase effect has

been obtained with 0.005 ml. of a partially purified yeast hexokinase containing 5.75 mg. of N per ml. Fluoride in concentrations ranging from 0.005 to 0.03 M consistently decreases the rate of oxygen consumption and usually increases the absolute amount of phosphate esterified.

Fructose and mannose are as suitable phosphate acceptors as glucose, while galactose and arabinose are inactive, as would be anticipated from the known properties of purified yeast hexokinase (11). Since fructose-6-phosphate has also been found to be unsatisfactory as an acceptor, it may be concluded that the cyclophorase and hexokinase preparations used in these experiments contain no significant phosphohexokinase activity. Table V summarizes the pertinent data establishing the nature of the phosphate esters formed during  $\alpha$ -ketoglutarate oxidation in the presence of yeast hexokinase. When either glucose or fructose was added in various limiting quantities, the total inorganic phosphate which disappeared exceeded the amount of hexose added by approximately 6 to 10 micromoles. This excess of esterified phosphate was completely hydrolyzed by N HCl at 100° in 7 minutes and was shown to consist for the most part of inorganic pyrophosphate. The product remaining after 7 minutes acid hydrolysis appears to be an equilibrium mixture of glucose- and fructose-6-phosphate. Isolated by barium and alcohol fractionation, the final product liberated 26.5 per cent of its total phosphorus as inorganic phosphate on acid hydrolysis at 100° for 180 minutes, a value which corresponds fairly closely to that calculated (30.8 per cent) for such an equilibrium mixture. The yeast hexokinase preparation contains the phosphohexose isomerase which catalyzes the equilibration of the hexose phosphates.

Glycerol has been shown to act as a phosphate acceptor during aerobic oxidation in homogenates of rabbit kidney (12), as have adenosine in dialyzed kidney extracts (13) and creatine in a washed mince of heart muscle (14) and homogenates of rat liver (15). Our attempts to use these substances as acceptors have been uniformly unsuccessful. The reactions by which they are phosphorylated apparently require enzymes which are either absent or inactive in the cyclophorase preparations at the third residue stage.

*Phosphatase Activity*—Three types of phosphatase activity have been examined; viz., the dephosphorylation of (1) hexose phosphate, (2) adenosine triphosphate, and (3) inorganic pyrophosphate. Under the conditions of active phosphorylation, neither glucose- nor fructose-6-phosphate is hydrolyzed at an appreciable rate. Fluoride in 0.01 M concentration only partially suppresses the adenosinetriphosphatase and inorganic pyrophosphatase of the cyclophorase preparation. Consequently, it is difficult to assess the extent to which these phosphatases interfere with maximal phosphorylation. The yeast hexokinase preparation contains no adeno-

sinetriphosphatase and its inorganic pyrophosphatase is completely inhibited by 0.01 M fluoride.

*Phosphate-Oxygen Ratio*—Considerable significance attaches to the determination of which oxidative reactions involve the esterification of inorganic phosphate and to the number of phosphate molecules esterified per atom of oxygen absorbed. There is an extensive literature on aerobic phosphorylation in animal tissues, some of the most notable contributions being those of Kalckar (12, 16), Belitzer and Tsibakova (14), Colowick *et al.* (13), Potter (15), and Ochoa (17, 18). These investigators have established

TABLE V  
*Phosphorylation with Limiting Amounts of Hexose*

Hexose added	Total disappearance of inorganic phosphate	Non-hydrolyzable P	Non-hydrolyzable P Hexose added	Fructose recovered	Final fructose Hexose added
	micromoles	micromoles		micromoles	per cent
0	8.0	1.0			
Fructose, 10 micromoles	20.4	11.1	1.01	3.3	33
“ 20 “	28.6	19.1	0.90	6.3	32
“ 30 “	39.8	29.9	0.96	9.4	31
“ 40 “	48.1	37.9	0.92	12.5	31
0	9.4	1.5			
Glucose, 10 micromoles	18.3	11.5	1.00	3.2	32
“ 20 “	28.2	20.4	0.95	6.6	33
“ 30 “	36.2	29.0	0.92	10.3	34
“ 40 “	46.4	39.5	0.95	13.1	33

Each vessel contained 1 ml. of  $R_3K$ , 0.5 ml. of 0.1 M phosphate buffer of pH 7.3, 0.3 ml. of 0.01 M adenosine-5-phosphate, 0.2 ml. of 0.1 M magnesium chloride, 0.1 ml. of 0.33 M fluoride, 0.3 ml. of 0.1 M  $\alpha$ -ketoglutarate, 0.02 ml. of yeast hexokinase, the amount of hexose indicated, and water to a final volume of 2.8 ml.; oxygen in the gas space. Temperature, 25°; time, 30 minutes. The oxygen uptake averaged 25 microatoms per vessel. The non-hydrolyzed P represents the esterified phosphate which resisted 7 minutes hydrolysis in N HCl at 100°. Fructose was estimated by the resorcinol method (39).

the linkage of phosphate esterification with the aerobic oxidation of pyruvate, citrate,  $\alpha$ -ketoglutarate, succinate, malate, oxalacetate, and glutamate. Ochoa (17) observed P:O ratios as high as 2.5 during the aerobic oxidation of pyruvate by a washed mince of cat heart. In the malonate-inhibited system catalyzing the one-step oxidation of  $\alpha$ -ketoglutarate to succinate (18), the observed P:O ratio was 1.7.

Certain difficulties arise in the estimation of the P:O ratio, owing to the instability of various enzymes of the cyclophorase complex in the absence of substrate. Consequently, the usual procedure of tipping in substrate at the end of an equilibration period is undesirable. The ratios to be reported



were measured by two alternative methods. In the *extrapolation* method, the components of the reaction were introduced into manometer vessels at 0°, the gas space quickly filled with oxygen, and the vessels were equilibrated with shaking in a 25° bath. The taps were closed at the end of 5 minutes and the oxygen uptake recorded for an additional 10 to 30 minutes, after which the reaction was terminated by precipitation with trichloroacetic acid. The oxygen uptake was corrected to include the equilibration period on the basis of the rate recorded during the first 5 minutes. Determinations by the *interval* method were accomplished by precipitat-

TABLE VI  
*Ratios of Phosphate Esterified to Oxygen Consumed with Different Substrates*

Substrate	Method of determination	No. of experiments	P:O ratios		
			Range	Mean	Standard deviation
$\alpha$ -Ketoglutarate	Extrapolation	62	1.51-3.43	2.43	$\pm 0.425$
	Interval	5	2.15-2.46	2.26	
Succinate	Extrapolation	26	0.88-1.55	1.25	$\pm 0.179$
	Interval	3	1.14-1.40	1.28	
Malate	Extrapolation	2	2.15-2.35	2.25	
	Interval	4	2.16-2.66	2.42	
Oxalacetate	"	3	1.94-2.20	2.06	
Pyruvate	Extrapolation	2	2.35-2.59	2.47	
Citrate	"	22	0.65-3.26	2.15	$\pm 0.648$
	Interval	3	1.92-2.99	2.30	
Proline	Extrapolation	20	1.41-2.91	2.07	$\pm 0.392$
	Interval	3	1.69-2.26	1.97	
Glutamate	Extrapolation	19	1.74-2.88	2.18	$\pm 0.314$
	Interval	2	2.14-2.46	2.30	

The manometer vessels were filled as described in Table IV. 30 micromoles of substrate were used except in the case of succinate, for which the added amount was 100 micromoles. The third residue from either rabbit kidney or rabbit liver was used in all experiments. Temperature, 25°; time, 15 to 35 minutes.

ing the contents of duplicate blank and experimental vessels at the beginning and end of the period of measured oxygen uptake. The P:O ratios as measured by the two methods checked well, except in occasional experiments of short duration in which the extrapolation correction constituted a large part of the estimated total oxygen uptake. The P:O ratios obtained with various substrates are summarized in Table VI.

A major problem in determining the P:O ratio for individual oxidative steps arises from the difficulty encountered in isolating the individual reactions. A variable and frequently considerable portion of the oxygen consumed is not accounted for by the disappearance of the added substrate

and must represent subsequent oxidative reactions (*cf.* Table VII). The use of supposedly specific inhibitors, such as malonate, is not entirely satisfactory in that such agents frequently depress the activity of the phosphorylation system. However, the data in Table VI suggest that more than 2 molecules of phosphate are esterified for each atom of oxygen absorbed in the various oxidative steps of the citric acid cycle, except that of succinate to fumarate, which consistently has a P:O ratio of less than 2. The oxidation of proline or glutamate also yields ratios in excess of 2. Since presumably only whole numbers of phosphate molecules can be esterified per atom of oxygen, it follows in those cases in which the experimentally determined ratio exceeds 2 that the probable ratio is 3 and is not due to back reactions. The maximal energy which can be obtained from the complete oxidation by molecular oxygen of a mole of pyruvate to carbon dioxide and water has been calculated to be 280 kilocalories (19).

TABLE VII  
*Disappearance of Added Substrates during Phosphorylation Experiments*

Substrate added	Disappearance of substrate	Oxygen uptake	Disappearance of inorganic phosphate	P:O	Micromoles substrate Microatoms O
	<i>micromoles</i>	<i>microatoms</i>	<i>micromoles</i>		
Citrate . . . . .	4.2	12.0	21.9	1.82	0.35
$\alpha$ -Ketoglutarate . . . . .	5.6	10.2	22.7	2.23	0.55
Succinate . . . . .	16.0	18.5	21.7	1.17	0.87

These experiments were performed in duplicate by the interval method. The additions were as described in Table IV with 30 micromoles of the added substrate. Total time at 25°, 15 minutes. The citrate disappearance reported is maximal, since no correction was made for aconitase activity.

Assuming a value of 12 kilocalories as the energy of the pyrophosphate bonds in adenosine triphosphate, approximately 24 such bonds are theoretically obtainable. The data presented above indicate that 13 to 14 energy-rich phosphate bonds may be formed during the five oxidative reactions of the citric acid cycle whereby pyruvate is completely oxidized. The efficiency of the system, therefore, approaches 60 per cent of the theoretical maximum.

The estimation of P:O ratios during fatty acid oxidation is complicated by the need for the simultaneous oxidation of some member of the citric acid cycle and also by the sensitivity of the fatty acid oxidases to fluoride. Recent studies by Lehninger and Kennedy (20), utilizing radioactive P<sup>32</sup>, have established the coupling of fatty acid oxidation and phosphorylation. Similar studies in this laboratory have confirmed their observations.

*Phosphorylation during One-Step Oxidation-Reductions*—Oxygen may be

replaced by ferricyanide as the oxidant for various reactions catalyzed by the cyclophorase system (1). The question arises whether the linkage between oxidation and phosphorylation also obtains when ferricyanide is used as the oxidant and, in addition, whether more than 1 mole of phosphate is esterified for each mole of substrate oxidized by 2 moles of ferricyanide. Ferricyanide in the concentration (0.033 M) necessary to carry out the experiments visibly alters the physical characteristics of the cyclophorase preparation, and, consequently, there is the possibility that certain enzymatic activities have been altered. In any event, the ratio of the moles of phosphate esterified to 2 moles of ferricyanide reduced is less than 1, as shown in Table VIII.

TABLE VIII  
*Phosphorylation in Anaerobic Oxidation by Ferricyanide*

Substrate	Ferricyanide reduced	Disappearance of inorganic phosphate	$P:\frac{1}{2}Fe(CN)_6^{3-}$
	micromoles	micromoles	
Succinate.....	61.2	5.7	0.19
Malate.....	37.9	11.9	0.63

The experimental manometer vessels contained 1 ml. of  $R_3L$ , 0.02 ml. of yeast hexokinase, 0.4 ml. of 0.125 M phosphate buffer of pH 7.3, 0.3 ml. of 0.01 M adenosine triphosphate, 0.2 ml. of 0.1 M magnesium chloride, 0.1 ml. of 1.0 M fructose, 0.2 ml. of 0.9 M sodium fluoride, 0.1 ml. of 0.5 M sodium bicarbonate, 0.2 ml. of 0.5 M potassium ferricyanide, 100 micromoles of succinate or 30 micromoles of malate, and water to a final volume of 3.0 ml. The gas space was filled with 5 per cent carbon dioxide in nitrogen. Total time at 38°, 30 minutes. Ferricyanide was estimated colorimetrically by its absorption at 420 m $\mu$  in the trichloroacetic acid filtrates. Inorganic phosphate was precipitated from the trichloroacetic acid filtrates by barium at pH 9.0, redissolved in hydrochloric acid, and estimated after removal of the barium with sulfate. A standard solution treated similarly gave a 97 per cent recovery of the added phosphate.

The anaerobic oxidation of various substrates can be coupled with the simultaneous reduction of a second substrate. Thus  $\alpha$ -ketoglutarate can be oxidized to succinate coincident with the reduction of acetoacetate to  $\beta$ -hydroxybutyrate. The esterification of inorganic phosphate which occurs during this anaerobic oxidation-reduction is shown in a representative experiment in Table IX. The experimentally determined ratio, moles of phosphate esterified to moles of  $\alpha$ -ketoglutarate oxidized, rarely exceeds a value of 0.6 under the conditions specified.

*Inorganic Phosphate Requirement*—Inorganic phosphate has been shown to be required for the activity of the  $\alpha$ -ketoglutaric dehydrogenase of cat heart by Ochoa (17) and for the fatty acid oxidase of rat liver by Lehnin-

ger and Kennedy (20). A similar phosphate requirement has been established for a number of the oxidative reactions catalyzed by the cyclophorase system. It has been impossible to achieve a zero concentration of inorganic phosphate in the reaction mixture because of the continuous liberation of small amounts of phosphate from the enzyme. Fig. 1 shows the relationship between the concentration of inorganic phosphate and the rate of  $\alpha$ -ketoglutarate oxidation. The decrease in the rate of oxygen consumption which occurs as available inorganic phosphate is removed from the reaction mixture by a suitable acceptor, such as the hexokinase-fructose system, is illustrated in Fig. 2. Even with this device, it has not been possible to reduce the level of inorganic phosphate below 0.2 micromole per ml. However, it appears evident that inorganic phos-

TABLE IX  
*Phosphorylation in Anaerobic Oxidation-Reduction*

	Carbon dioxide evolved	Disappearance of acetoacetate	Disappearance of inorganic phosphate	$\frac{P}{\text{Acetoacetate}}$
	<i>micromoles</i>	<i>micromoles</i>	<i>micromoles</i>	
No substrate.....	0			
" $\alpha$ -ketoglutarate.....	0.1	2.2	0.7	
" acetoacetate.....	2.0		2.3	
" fluoride.....	9.8	9.0	-0.4	
Complete system.....	9.6	9.9	6.1	0.62

The complete system consisted of 1.5 ml. of  $R_2K$ , 0.02 ml. of yeast hexokinase, 0.2 ml. of 0.1 M phosphate buffer of pH 7.3, 0.3 ml. of 0.01 M adenosine-5-phosphate, 0.2 ml. of 0.1 M magnesium chloride, 0.1 ml. of 0.33 M sodium fluoride, 0.1 ml. of 1.0 M fructose, 0.2 ml. of 0.1 M sodium bicarbonate, 0.3 ml. of 0.1 M  $\alpha$ -ketoglutarate, 0.3 ml. of 0.1 M acetoacetate, and water to a final volume of 3.1 ml.; 5 per cent carbon dioxide in nitrogen in the gas space. The other manometer vessels were identical except for the indicated omissions.

phate is an essential component of the reaction and that a concentration between 1 and 2 micromoles per ml. is needed for maximal activity. Data which indicate that inorganic phosphate is required for various oxidation reactions catalyzed by the cyclophorase system are presented in Tables X and XI.

*Adenylic Acid Requirement*—Numerous investigators have observed that the addition of adenosine-5-phosphate is essential for restoring the oxidative activity of various washed and dialyzed animal tissue residues. An adenylic acid requirement has been established for all but a few of the oxidation reactions catalyzed by the cyclophorase system (*cf.* Table XII for representative data). In the oxidation of succinate to fumarate and proline to glutamic semialdehyde (5), the rate of substrate disappearance is completely independent of the presence of added adenylic acid. How-

ever, even in these instances, little or no phosphorylation of hexose occurs unless adenylic acid is added to the oxidizing system. Thus, adenylic acid serves in two capacities: (1) that of restoring the activity of various oxidative enzymes, and (2) as a catalytic carrier between the phosphate donor and acceptor systems. In order to serve in the first capacity, adenylic acid

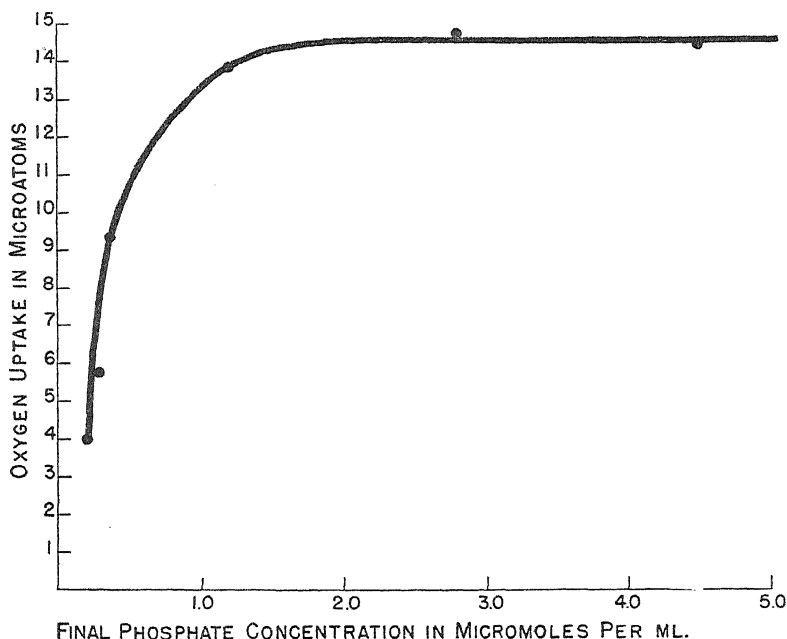


FIG. 1. Phosphate requirement for  $\alpha$ -ketoglutarate oxidation. Each manometer vessel contained 1 ml. of  $R_3K$ , 0.3 ml. of 0.01 M adenosine-5-phosphate, 0.2 ml. of 0.1 M magnesium chloride, 0.1 ml. of 0.1 M fluoride, 0.3 ml. of 0.1 M  $\alpha$ -ketoglutarate, 0.9 per cent potassium chloride to a final volume of 2.8 ml., and oxygen in the gas space. In addition, the following amounts of inorganic phosphate of pH 7.3 were added to successive cups: 0, 3, 6, 10, 15, and 20 micromoles. The abscissa represents the final inorganic phosphate concentration. The ordinate represents oxygen uptake in the first 15 minutes after a 5 minute equilibration period at 25°.

must combine with the apooxidase, whereas in the second it is presumably free in solution.

Adenylic acid can be replaced quantitatively by either adenosine diphosphate or adenosine triphosphate, since all three forms are interconvertible in the enzyme complex. Inosinic acid and an unusual adenosine polyphosphate recently isolated from mung beans by Albaum *et al.* (21) can also replace adenylic acid. Adenosine, yeast adenylic acid, and guanylic acid are inactive in restoring either oxygen consumption or phosphorylation.

A preparation of cozymase of approximately 75 per cent purity partially replaces adenylic acid in the phosphorylation system, but there is no summation of effects when both nucleotides are present. Alkali-inactivated cozymase has the same restorative effect, a finding which suggests that

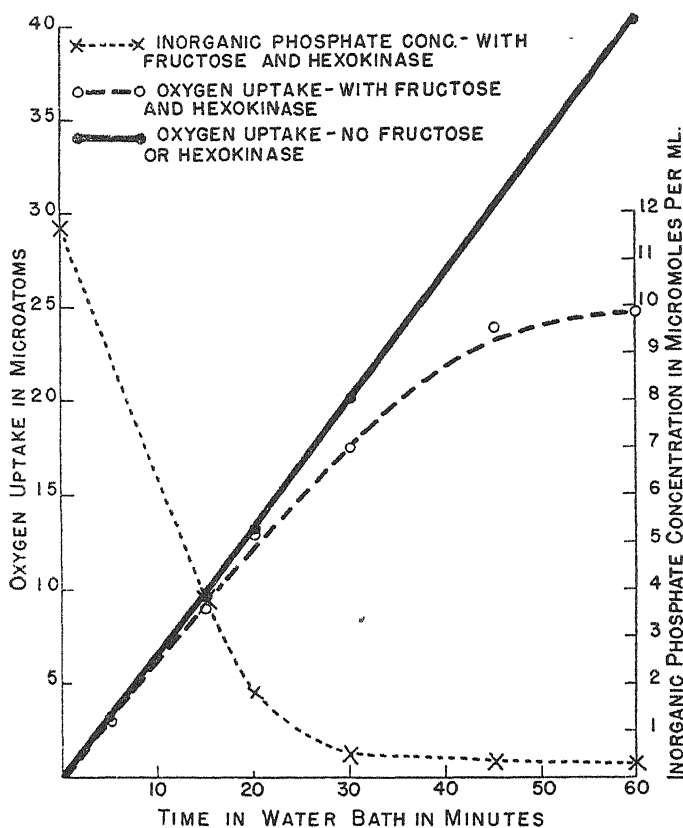


FIG. 2. Phosphate requirement for  $\alpha$ -ketoglutarate oxidation. Five manometer vessels were set up as described in the legend below Table IV, but with 0.3 ml. of 0.1 M phosphate buffer. A sixth was identical except for the omission of hexokinase and fructose. A seventh served as a blank with no substrate. The vessels containing hexokinase and fructose were removed from the water bath at the times indicated and the final inorganic phosphate concentration determined. The temperature of the water bath was 25°.

cozymase is active by virtue of its ability to undergo cleavage to yield adenylic acid, as recently described in kidney residues by Kornberg and Lindberg (22). Various coenzyme II preparations of low purity have been observed to produce similar effects, but it is possible that these results are

TABLE X  
Phosphate Requirement of Various Oxidation Reactions

Experiment No.	Substrate	No hexokinase or sugar		With hexokinase and sugar	
		Final phosphate concentration	Oxygen uptake	Final phosphate concentration	Oxygen uptake
		<i>micromoles per ml.</i>	<i>c.mm.</i>	<i>micromoles per ml.</i>	<i>c.mm.</i>
1	$\alpha$ -Ketoglutarate	3.9	135	0.4	73
2	Succinate	5.0	136	0.4	88
3	Malate	4.3	102	0.4	54
3	Oxalacetate	2.9	93	0.5	40
4	Citrate	4.0	128	0.3	45
4	Glutamate	4.3	87	0.5	31

The manometer vessels contained 1 ml. of  $R_2K$ , 0.5 ml. of 0.04 M phosphate buffer of pH 7.3, 0.3 ml. of 0.01 M adenosine-5-phosphate, 0.2 ml. of 0.1 M magnesium chloride, 0.1 ml. of 0.33 M sodium fluoride, 0.3 ml. of 0.1 M substrate solution (except in the case of succinate when 0.2 ml. of 0.5 M solution was used), water to a final volume of 3.0 ml., and oxygen in the gas space. Each substrate was set up in duplicate, one cup containing in addition to the above components 0.02 ml. of yeast hexokinase and 0.1 ml. of 1.0 M fructose. All experiments were done at 25°. The oxygen figures represent the uptake during the last 10 minutes of a 30 minute experiment.

TABLE XI  
Phosphate Requirement for Fatty Acid Oxidation

Substrates added	Final inorganic phosphate concentration	Oxygen uptake
	<i>micromoles per ml.</i>	<i>c.mm.</i>
$\alpha$ -Ketoglutarate (2 micromoles).....	0.8	83
Same.....	7.6	86
" + butyrate (30 micromoles).....	0.9	185
" + " (30 " ).....	7.9	399
" + $\beta$ -ketohexanoate (30 micromoles).....	1.1	161
" + " (30 " ).....	7.1	400

Each vessel contained 1.0 ml. of  $R_2K$ , 0.3 ml. of 0.01 M adenosine-5-phosphate, 0.2 ml. of 0.1 M magnesium chloride, substrates as indicated. Alternate vessels contained 0 or 0.2 ml. of 0.1 M phosphate of pH 7.3. Final volume 2.8 ml. Oxygen uptake values represent a 30 minute period at 25°.

attributable to contaminating amounts of adenylic acid. With one co-enzyme II preparation of relatively high purity (55 per cent), the restorative effects were lacking.

The addition of various other known coenzymes, such as diphosphothiamine, flavin-adenine dinucleotide, pyridoxal phosphate, and cytochrome *c*,

to the complete system does not alter the rate of either oxygen consumption or phosphorylation.

*Linkage of Phosphate Esterification with Succinate Oxidation*—The enzyme which catalyzes the oxidation of succinate to fumarate is the hardest of the oxidation enzymes in the cyclophorase system. Incubation of the complex for 15 minutes at 38° in the absence of substrate completely inactivates the  $\alpha$ -ketoglutarate- and malate-oxidizing enzymes, but leaves the succinoxidase activity unimpaired. This quality of the succinic enzyme has been useful in uncovering other features of the linkage between oxidation and

TABLE XII  
*Adenylic Acid Requirements of Different Substrates*

Substrate	Oxygen uptake	
	Without adenylic acid	With adenylic acid
	<i>c.mm.</i>	<i>c.mm.</i>
Pyruvate.....	119	341
Citrate.....	71	216
$\alpha$ -Ketoglutarate.....	88	224
Succinate.....	280	288
Malate.....	38	94
Glutamate.....	43	155
Butyrate.....	115	260

Each vessel contained 1 ml. of an  $R_3K$  previously aged for 5 hours at 0°, 0.5 ml. of 0.1 M phosphate buffer of pH 7.3, 0.2 ml. of 0.1 M magnesium chloride or sulfate, 0.3 ml. of 0.1 M substrate (except succinate of which 0.2 ml. of 0.5 M was added), 0.3 ml. of 0.01 M adenosine-5-phosphate as indicated, water to a final volume of 2.8 ml., and oxygen in the gas space. In addition, the pyruvate cups contained 0.1 ml. of 0.5 M sodium bicarbonate and those with butyrate contained 0.2 ml. of 0.01 M  $\alpha$ -ketoglutarate. Temperature 25°, except for pyruvate and butyrate, for which it was 38°. The oxygen uptakes are those recorded during the first 15 minutes after a 5 minute equilibration period and have been corrected by subtraction of the appropriate blanks.

phosphorylation. With progressively longer exposure of the enzyme to a temperature of 38° before the addition of substrate, the P:O ratio becomes progressively smaller, while the rate of succinate oxidation remains unchanged (*cf.* Table XIII).<sup>2</sup> This effect can be duplicated with 0.02 M arsenite or 0.003 M phlorhizin. The inhibition of phosphorylation occurs despite a sustained rate of succinate oxidation. Thus, it must be concluded that prior incubation of the enzyme at 38°, arsenite, and phlor-

<sup>2</sup> Hexokinase was present in sufficient excess so that a slight loss of its activity during the more prolonged incubation periods did not make it a limiting factor in the over-all reaction.



hizin are capable of inactivating the mechanism whereby inorganic phosphate is transferred to adenylic acid to form adenosine triphosphate coincident with the oxidation of succinate.

*Gramicidin and Dinitrophenol*—The studies of Hotchkiss (23) and others have implicated gramicidin and 2,4-dinitrophenol as reagents capable of interrupting the normal linkage of oxidation and phosphorylation in aerobic systems. Both agents stimulate the respiration of staphylococci, but at the same time block the uptake of inorganic phosphate from the medium which normally accompanies respiration. Dinitrophenol, but not gramicidin, acts similarly on respiring yeast cells. In unpublished experiments mentioned in his review article (24), Hotchkiss found that gramicidin blocks

TABLE XIII  
*Effect on Phosphorylation of Preheating Enzyme at 38°*

Time at 38° without substrate	Oxygen uptake	Disappearance of inorganic phosphate	P:O
<i>min.</i>	<i>microatoms</i>	<i>micromoles</i>	
0	27.5	24.8	0.90
5	27.8	24.6	0.88
15	28.7	21.9	0.76
25	33.5	18.0	0.54
45	31.0	5.6	0.18

The manometer vessels were filled as described in Table IV, except that 0.2 ml. of 0.5 M succinate was placed in the side arm and was tipped in after the indicated number of minutes of shaking in the water bath at 38°. The values for zero minute were obtained in a separate experiment performed with the same enzyme preparation by the interval method described in the text. Time of experiment after tipping in substrate, 15 minutes.

the phosphorylation of glucose by cell-free kidney extracts without altering the rate of oxygen consumption.

Tested in the cyclophorase system, both gramicidin and 2,4-dinitrophenol in high dilution effectively inhibit oxidative phosphorylation without depressing the rate of  $\alpha$ -ketoglutarate oxidation (*cf.* Table XIV). An apparent stimulation of respiration is frequently observed, but such stimulation is most consistently encountered in fluoride-containing systems and may represent a partial reversal of the inhibitory effect of fluoride on respiration. With other substrates, such as succinate and malate, the respiratory effects are variable and depression of oxygen uptake occurs frequently, even at high dilutions of the inhibitors, independently of the presence of fluoride.

Two somewhat more soluble derivatives of gramicidin, *viz.* methylol-gramicidin (25) and the succinyl half ester (26), have approximately the

same activity as the parent compound. Tyrocidine in concentrations as high as 100  $\gamma$  per ml. (approximately  $1$  to  $3 \times 10^{-5}$  M) is completely inactive, while higher concentrations depress both oxidation and phosphorylation to a comparable degree.

A high degree of structural specificity underlies the action of 2,4-dinitrophenol, as shown in Table XV, which demonstrates certain limits of change which are permissible in the structural configuration of the inhibitor. From the limited data available, it may be concluded that the phenolic group is indispensable for inhibitory activity. Substitution in

TABLE XIV  
*Effect of Gramicidin and Dinitrophenol on Phosphorylation*

Inhibitor	Concentration	Oxygen uptake	Disappearance of inorganic phosphate	P:O
	$\times 10^{-6}$ M	microatoms	micromoles	
Gramicidin	0.0	13.9	30.2	2.17
	0.0017*	13.3	30.7	2.30
	0.02	17.0	21.9	1.29
	0.25	18.7	13.7	0.73
	3.0	16.6	9.4	0.57
	36.0†	18.7	6.9	0.37
2,4-Dinitrophenol	0.0	14.2	32.6	2.30
	0.4	15.4	33.8	2.20
	2.0	16.3	31.2	1.91
	10.0	16.8	22.0	1.31
	50.0	15.6	6.5	0.42
	250.0	13.9	2.5	0.18

Experiments were performed as described in Table IV. Gramicidin (methylol) was added in 0.02 ml. of 95 per cent alcohol, the dinitrophenol in aqueous solution. The duration of the experiment at 25° was 20 and 25 minutes respectively.

\* Molecular weight of gramicidin assumed to be 2800.

† Approximate limit of solubility of gramicidin.

the 6 position of 2,4-dinitrophenol with methyl, isobutyl, or certain saturated and unsaturated cyclic constituents tends to enhance inhibitory activity, whereas a carboxyl or third nitro group yields compounds which are completely inactive at  $2 \times 10^{-4}$  M concentration. Replacement of the 4-nitro group of picrate with *n*-octyl or chloro groups partially restores the inhibitory activity. A rather striking parallelism exists between the activity of the various nitrophenols reported by Clowes and Krah1 (27) to stimulate the respiration and inhibit the cell division of fertilized *Arbacia* eggs and their activity in the cyclophorase system. The active dihalo- and trihalophenols reported by the same investigators (28) have not been ex-

**TABLE XV**  
*Effect of Nitrophenols and Related Compounds on Phosphorylation*

Substituted benzene compounds					Inhibition of phosphorylation
Position					
1	2	3	4	6	
OH	NH <sub>2</sub>				0
"			NH <sub>2</sub>		0
"	NH <sub>2</sub>		"		0
"	NO <sub>2</sub>				0
"		NO <sub>2</sub>			0
"			NO <sub>2</sub>		0
"	NH <sub>2</sub>		"		0
"	NO <sub>2</sub>		"		++++
"	"		"	NH <sub>2</sub>	++
"	"		"	CH <sub>3</sub>	++++
"	"		"	C <sub>6</sub> H <sub>5</sub> *	++++
"	"		"	C <sub>6</sub> H <sub>5</sub>	++++
"	"		"	C <sub>6</sub> H <sub>11</sub> †	++++
"	"		"	COOH	0
"	"		"	NO <sub>2</sub>	0
OCH <sub>3</sub>	"		"		0
OC <sub>2</sub> H <sub>5</sub>	"		"		0
COOH	"		"		0
SO <sub>3</sub> H	"		"		0
CH <sub>2</sub> COOH	"		"		0
NH <sub>2</sub>	"		"		0
Cl	"		"		0
OH	"	OH			0
"	"	"	NO <sub>2</sub>		0
"	"	"	"	NO <sub>2</sub>	0
"	"		CH <sub>3</sub>		0
"	"		C <sub>6</sub> H <sub>11</sub> †	NO <sub>2</sub>	+++
"	"		Cl	"	++++
"	Cl		"		0
"	"		NO <sub>2</sub>	Cl	++++
"			Br		0
Miscellaneous compounds					
2-Nitro-1-naphthol					0
2,4-Dinitro-1-naphthol					++++
2,2'-Methylenebis(4-nitrophenol)					++
2,2'-Methylenebis(4-chlorophenol)					±
2,2'-Methylenebis(4-bromophenol)					±
Bis(3,5,6-trichloro-2-hydroxyphenyl)methane					++++

The additions to the manometer vessels were as described in Table IV. The final concentration of the various agents was  $2.5 \times 10^{-4}$  M. The degree of inhibition is an expression of the relative lowering of the P:O ratio.

\* Secondary butyl.

† Cyclohexyl.

aminated in any systematic fashion, but two of the compounds listed in Table XV are more active than dinitrophenol when tested at low concentrations.

In a recent preliminary note, Loomis and Lipmann (29) have also reported the inhibition of oxidative phosphorylation by dinitrophenol in a kidney cyclophorase system oxidizing glutamate. They have made the additional interesting observation that minute amounts of dinitrophenol can apparently "replace" inorganic phosphate in this reaction. This finding has since been confirmed by us. The oxidation of  $\alpha$ -ketoglutarate pro-

TABLE XVI

*Effect of Gramicidin and Dinitrophenol on Fatty Acid Oxidation*

Substrate	Oxygen uptake		
	No inhibitor	With gramicidin	With dinitrophenol
	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>
Acetate.....	191	10	3
Butyrate.....	458	20	4
Acetoacetate.....	203	33	21
$\beta$ -Ketooctanoate.....	232	-5	-1
Isocrotonate.....	243	15	8

The experimental manometer vessels contained 1 ml. of  $R_3K$ , 0.5 ml. of 0.1 M phosphate buffer of pH 7.3, 0.3 ml. of 0.01 M adenosine-5-phosphate, 0.2 ml. of 0.1 M magnesium chloride, 0.2 ml. of 0.01 M  $\alpha$ -ketoglutarate (except in the case of acetoacetate, when 0.2 ml. of 0.025 M  $\alpha$ -ketoglutarate was used), 0.3 ml. of 0.1 M solution of the neutralized fatty acid indicated (except for  $\beta$ -ketooctanoate in which 0.1 ml. of 0.1 M solution was used), 0.02 ml. of 1.4 per cent gramicidin in 75 per cent ethyl alcohol or 0.2 ml. of 0.0014 M dinitrophenol as indicated, and water to a final volume of 2.8 ml.; alkali in the center well and oxygen in the gas space. The vessels were shaken in the water bath at 38° for 20 minutes (35 minutes in the case of acetoacetate) to permit the bulk of the  $\alpha$ -ketoglutarate to be oxidized, and oxygen uptakes were recorded for the next 30 minutes. The oxygen uptake figures were obtained after subtraction of the relatively small values obtained simultaneously in vessels containing the appropriate inhibitor and all other components except the fatty acid.

ceeds at normal or accelerated rates in the presence of  $5 \times 10^{-5}$  M dinitrophenol or  $3 \times 10^{-5}$  M gramicidin with inorganic phosphate concentrations as low as 0.2 micromole per ml. (*cf.* Fig. 1).

In previous communications (2, 3), evidence was presented to show that the oxidation of fatty acids,  $\beta$ -unsaturated acids, or  $\beta$ -keto acids proceeds only with the simultaneous oxidation of some member of the citric acid cycle. In view of the probability that this obligatory cooxidation involves a phosphorylation reaction, we have examined the effects of gramicidin and dinitrophenol on fatty acid oxidation. Both reagents block the oxidation

of acetate, butyrate, acetoacetate,  $\beta$ -ketooctanoate, and isocrotonate despite the fact that the oxidation of the citric acid cycle substrate ( $\alpha$ -ketoglutarate) is essentially unchanged (*cf.* Table XVI). Although the possibility remains that gramicidin and dinitrophenol are not limited in their action to the inhibition of phosphorylation, the findings presented suggest that it is the phosphorus transformation accompanying cooxidation of the cycle substrate which underlies the activation of fatty acids. However, in this connection it is interesting to note that Clifton (30) has observed that dinitrophenol in appropriate concentrations ( $1.25$  to  $2.5 \times 10^{-4}$  M) appears to promote the more complete oxidation of acetate and butyrate added to suspensions of *Pseudomonas calco-acetica* in phosphate buffer of pH 7.0. Insufficient information is at hand to justify attempts to explain these apparently conflicting observations.

#### DISCUSSION

The conclusions permissible on the basis of the present study of oxidative phosphorylation in the cyclophorase system may be summarized as follows: (1) Every oxidative reaction catalyzed by the cyclophorase and associated enzyme systems involves esterification of inorganic phosphate. (2) For each atom of oxygen absorbed from 2 to 3 molecules of inorganic phosphate are esterified. (3) There is no evidence that acyl phosphates are formed in any of these reactions. (4) The first product of orthophosphate esterification (primary ester) must be capable of yielding inorganic pyrophosphate, since inorganic pyrophosphate is formed as a decomposition product in each of the oxidative phosphorylation reactions. (5) In the phosphorylation of hexose at least two steps can be recognized: the transfer of phosphate from the primary ester to adenylic acid (catalyzed by the cyclophorase system) and then the transfer of phosphate from adenosine triphosphate to hexose (catalyzed by hexokinase). (6) Gramicidin and dinitrophenol are reagents which prevent the transformation of inorganic phosphate into hexose phosphate during the operation of the cyclophorase system and to the same degree they eliminate all the various reactions involved in fatty acid oxidation which depend upon the simultaneous oxidation of a substrate of the cyclophorase system. (7) Finally, the oxidative enzymes of the cyclophorase and associated systems generally have the same pattern of requirements (inorganic phosphate, adenylic acid, magnesium ions) and exhibit properties which sharply differentiate them from the non-cyclophorase enzymes which catalyze some of the oxidative reactions of the citric acid cycle.

It has been possible on the basis of these experimental findings to formulate a mechanism which provides a satisfactory explanation of the data. In the following communications of this series various experiments will be

reported which offer direct confirmation of this mechanism. Hence only the barest summary of this proposed mechanism will be in order at this point. Each of the oxidative enzymes of the cyclophorase complex contains a coenzyme which is firmly bound to the protein moiety. These coenzymes are the instruments by which inorganic phosphate becomes esterified. As the coenzyme undergoes reduction, simultaneously inorganic phosphate becomes esterified and a coenzyme pyrophosphate is formed. Each of the primary coenzymes reacts with oxygen through a series of intermediary coenzymes which also have their cycles of oxidation-reduction linked with phosphate esterification. It follows that the esterification of inorganic phosphate cannot be dissociated from the oxidative reaction.<sup>3</sup> The esterification is an integral part of the process in the same sense that the reduction of the pyridine nucleotides is accompanied by the formation of an acid group. Finally the so called sparking reaction observed in fatty acid oxidation resolves itself into (a) the generation of the coenzyme pyrophosphate and (b) the formation of a complex between the coenzyme pyrophosphate and the fatty acid or fatty acid derivative. This complex and not the original fatty acid or fatty acid derivative is the substrate of the appropriate oxidase.

The presence of considerable amounts of various coenzymes bound to the protein of the cyclophorase preparation has been demonstrated. Thus far, evidence has been obtained for the presence of the following: (a) adenylic acid and adenosine polyphosphate, (b) coenzymes I and II, (c) flavin-adenine dinucleotide, (d) diphosphothiamine, and (e) the acetylation coenzyme discovered by Lipmann *et al.* (31) and Nachmansohn and Ber- man (32). No member of the cytochrome group has been directly identified, but the presence of cytochrome *c* is indicated indirectly by the demonstration of active *p*-phenylenediamine oxidation in the absence of added cytochrome. In addition, it has been observed that oxygen consumption can be markedly depressed by 0.03 M sodium azide, an effective inhibitor of cytochrome oxidase. As would be expected, sodium azide exhibits no depressant effect on oxidation under anaerobic conditions when ferricyanide serves as oxidant.

All but a few of the cyclophorase oxidases require the addition of adenylic acid for maximal activity and, consequently, the term *adenylo-proteins* has been applied to these enzymes. It is entirely possible that the other enzymes, such as succinoxidase and proline oxidase, are also adenyloproteins, but that their activity is maintained by adequate amounts of adenylic acid more firmly bound on the apoenzymes.

<sup>3</sup>The apparent exceptions to this correlation, such as the so called "replacement" of inorganic phosphate by dinitrophenol or the decline of the P:O ratio for the cyclophorase succinic oxidase system to a value approaching zero, will be considered in a subsequent communication.

The properties which serve to distinguish the oxidases associated with the cyclophorase system from their classical counterparts have been summarized on Table XVII. There is ample justification for not confusing the oxidative enzymes of the cyclophorase system with any of the classical oxidases previously described.

Five reactions catalyzed by the cyclophorase system have been shown previously (2, 3) to require the simultaneous oxidation of some member of the citric acid cycle: (a) the oxidation of fatty acid to unsaturated acid, (b) the oxidation of unsaturated acid to  $\beta$ -keto acid, (c) the cleavage of  $\beta$ -keto acid to acetic acid and the residue fatty acid, (d) the condensation of acetic and oxalacetic acids to form citric acid, and (e) the condensation of acetoacetic and oxalacetic acids to form citric acid. Reaction (e) may well be a combination of reactions (c) and (d). All five of these reactions are

TABLE XVII  
*Comparison of Classical and Cyclophorase Dehydrogenases*

Oxidase	Classical		Cyclophorase	
	Requirement	Ability to esterify inorganic P	Requirement	Ability to esterify inorganic P
Malic.....	DPN or TPN	0	AMP, Mg, P	+
Glutamic.....	" " "	0	" " "	+
Isocitric.....	TPN	0	" P	+
Succinic.....		0		+
$\alpha$ -Ketoglutaric.....	Diphosphothiamine	0	AMP, Mg, P	+
$\beta$ -Hydroxybutyric.....	DPN	0	" P	+

DPN = diphosphopyridine nucleotide; TPN = triphosphopyridine nucleotide; AMP = adenosine 5-phosphate.

sensitive to minute amounts of gramicidin and dinitrophenol, which suggests that the activation of fatty acids is somehow dependent upon the phosphorylation processes accompanying the oxidation of the citric acid cycle substrate. In addition, the oxalacetate arising from the citric acid cycle also provides the condensation partner for the two carbon fragments released from fatty acids by  $\beta$  oxidation.

#### *Methods and Materials*

*Isolation of Inorganic Pyrophosphate*—Approximately 100 ml. of the liver enzyme suspension ( $R_3L$ ) were mixed with 3.35 ml. of M sodium fluoride, 13.4 ml. of 0.02 M magnesium sulfate, 20 ml. of 0.01 M adenosine triphosphate, 20 ml. of 0.1 M  $\alpha$ -ketoglutarate, 20 ml. of a 1 per cent solution of heart acetone powder (*cf.* Knox *et al.* (3) for the details of preparation), and 33.5 ml. of 0.04 M phosphate buffer of pH 7.2. The mixture was

placed in a three-necked round bottom flask, gassed with oxygen, and incubated for 90 minutes with shaking in a bath maintained at 38°. At the end of this period the reaction mixture was cooled to 0° and treated with 66 ml. of cold 50 per cent trichloroacetic acid, cold water being added to a final volume of 660 ml. All subsequent manipulations were carried out in the cold. To the trichloroacetic acid filtrate was added 0.66 ml. of 20 per cent lead acetate and the pH was raised to 1.5 to 1.6 by the slow addition of alkali. The lead precipitate was centrifuged, redissolved in 5 per cent trichloroacetic acid, and reprecipitated at pH 1.2 in the presence of added lead acetate. The second lead precipitate was washed with water and after suspension in water was decomposed with hydrogen sulfide. The supernatant of the lead sulfide precipitate was brought to pH 6.0 with barium hydroxide and the barium salt was centrifuged, washed with water, and dried *in vacuo* at 100° over phosphorus pentoxide. The final powder (19.8 mg.) was light brown in color. For purposes of comparison, a sample of reagent grade sodium pyrophosphate was carried through the entire isolation procedure at the same time.

*Preparation of Enzymes*—The cyclophorase enzymes of rabbit kidney and liver were prepared as described in Paper I of this series (1). Yeast hexokinase, prepared by the method of Berger *et al.* (9), was carried through the second alcohol precipitation, the fraction obtained between 29 and 45 per cent alcohol being retained. A concentrated solution of the hexokinase in 1 per cent glucose was found to retain its activity for several months when kept frozen at -5°. The yeast hexokinase preparation served also as the source of inorganic pyrophosphatase. The properties of the enzyme found in this preparation correspond to those described by Bailey and Webb (7) for the inorganic pyrophosphatase prepared from yeast by a somewhat different procedure. A muscle hexokinase was prepared by the method of Cori and Slein (10).

*Methods*—The manometric procedures were performed in the usual manner or as described in the text and table legends. The following analytical methods were used: inorganic phosphate (33), fructose (34), pentose (35), succinate (36),  $\alpha$ -ketoglutarate (37), citrate (38), and acetoacetate (39).

*Sources of Special Chemicals*—Adenosine-5-phosphoric acid was obtained from the Ernst Bischoff Company of Ivoryton, Connecticut. Adenosine triphosphate was prepared by the method of LePage (40) or was supplied as the free acid by the Schwarz Laboratories. Inosinic acid was isolated from rabbit skeletal muscle by the methods of Ostern (41). Dr. Harry Albaum kindly gave us samples of adenosine diphosphate barium salt and the adenosine polyphosphate isolated by him from mung beans (21). Gramicidin and its formaldehyde and succinate derivatives



were generously supplied by the Wallerstein Laboratories. We are also indebted to Dr. C. J. Wessel, Assistant Director of the Prevention of Deterioration Center of the National Research Council, for most of the compounds listed in Table XV.

#### SUMMARY

Esterification of inorganic phosphate accompanies all of the oxidative steps catalyzed by the cyclophorase system. From 2 to 3 atoms of P are taken up for each atom of oxygen absorbed. Inorganic phosphate appears to be an essential requirement for each of the oxidative reactions. In the absence of a phosphate acceptor, the inorganic phosphate which disappears accumulates as inorganic pyrophosphate. Gramicidin and dinitrophenol paralyze esterification of inorganic phosphate without affecting the rate of oxidation to a comparable degree. These two reagents also paralyze the sparking of fatty acid oxidation.

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# THE DISTRIBUTION OF FIXED RADIOACTIVE CARBON IN GLUCOSE FROM RAT LIVER GLYCOGEN\*

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(Received for publication, August 13, 1948)

In a previous publication (1) it was reported that  $C^{13}$  administered to rats as bicarbonate could be detected only in the two center carbons of the glucose isolated from liver glycogen. Because of the great dilution of the isotope in the glucose, concentrations of  $C^{13}$  as high as 10 to 20 per cent of that found in carbons 3 and 4 could have been present in other positions of the glucose molecule and escaped detection by the mass spectrometer. Availability of  $C^{14}$ , because of the greater dilution tolerated by this isotope, has afforded an opportunity to reinvestigate the problem more critically. This has been done because observations on  $CO_2$  fixation are essential in interpreting results of experiments in which isotopic  $CO_2$  arises secondarily, and because of the general importance of  $CO_2$  fixation *per se*. Though the present experiments amply confirm the essential findings with  $C^{13}$ , they also indicate that a small amount of isotope, just detectable by present methods, and probably of the order of 1 to 2 per cent of that found in carbons 3 and 4 of the glucose, occurs in other positions of the glucose molecule.

## Methods

The plan of the experiments and the procedures employed were, in the main, those already described (1). Albino rats, after a 24 hour fast, were given glucose by stomach tube and isotonic  $NaHC^{14}O_3$  subcutaneously. After  $2\frac{1}{2}$  hours the animals were sacrificed and the liver glycogen isolated (2) and hydrolyzed to glucose. The pooled glucose from two animals was converted to lactic acid by fermentation with *Lactobacillus casei*, and the lactic acid oxidized with permanganate to yield carbons 3 and 4 of the original glucose as  $CO_2$ , and the balance of the molecule as acetaldehyde. In one experiment, the acetaldehyde was oxidized to  $CO_2$  with chromic acid (3), and, in another, it was further degraded by the iodoform reaction. In this reaction, carbons 2 and 5 of the original glucose are converted to

\* Aided by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

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formic acid, and carbons 1 and 6 to iodoform. The formic acid was oxidized to  $\text{CO}_2$  with mercuric acetate, and the iodoform with chromic acid.

All the fractions were converted to  $\text{BaCO}_3$  and the activity measured with a thin window Geiger-Müller tube. In one experiment the respiratory  $\text{CO}_2$  was collected over three 50 minute periods, and its activity measured. One control experiment was performed in which the animal received no isotope, but the  $\text{NaHC}^{14}\text{O}_3$  ( $5 \times 10^5$  counts per minute) was added to the tube in which the liver was being digested. This was done to determine whether or not the process of isolating the glycogen and hydrolysis to glucose succeeded in removing all the radioactive bicarbonate. Activity was determined directly on the glucose. None was found.

TABLE I

*Distribution of  $\text{C}^{14}$  in Glucose from Liver Glycogen*

The activity is measured in counts per minute per mg. of carbon.

Experiment No.	Rat weight	20 per cent glucose by stomach tube		Isotonic $\text{NaHC}^{14}\text{O}_3$ subcutaneously	Total activity of administered $\text{NaHC}^{14}\text{O}_3$	Activity of various fractions of glucose			Activity of respiratory $\text{CO}_2$		
		gm.	ml.			Carbons 3 and 4	Carbons 2 and 5	Carbons 1 and 6	1st 50 min.	2nd 50 min.	3rd 50 min.
1	210	6.0	3.0	$3 \times 10^5$	540	6	10				
	270	8.0	3.0	$3 \times 10^5$	$\pm 11$	$\pm 1.6$	$\pm 2$				
2	340	6.0	1.0	$1 \times 10^5$	138	$1.8 \pm 1.8$			851	388	230
					$\pm 3.5$				$\pm 8.9$	$\pm 6.1$	$\pm 5.5$
	290	8.7	1.0	$1 \times 10^5$					1441	244	89
									$\pm 12$	$\pm 5.2$	$\pm 3.3$

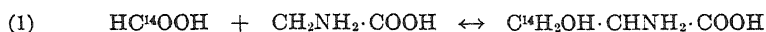
## RESULTS AND DISCUSSION

The principal results are summarized in Table I. In Experiment 1 the specific activity of the other positions is found to be 1 to 2 per cent of that of carbons 3 and 4. There is an apparently greater activity in positions 1 and 6 than in positions 2 and 5 but at the level of activity found in these experiments no significance can be attached to the differences. In Experiment 2, the activity recorded for carbons 1, 2, 5, and 6 is not significant, but, if taken at face value, bears approximately the same relationship to carbons 3 and 4 as noted in Experiment 1.

Possible mechanisms for the introduction of  $\text{CO}_2$  into the two center positions of the glucose molecule have been discussed (1). The formation of a 4-carbon dicarboxylic acid from the addition of  $\text{CO}_2$  and pyruvate cannot explain the appearance of isotope in any but carbons 3 and 4. Isotope could be introduced into the balance of the carbon chain by the presence of a 3-carbon symmetrical intermediate, such as dihydroxyacetone

which might be formed to a small extent by dephosphorylation of dihydroxyacetone phosphate. From this compound and the reversible reactions of glycolysis, pyruvate labeled in the methyl and carboxyl groups could be formed. Passage of this pyruvate through the Krebs cycle would randomize the isotope between the methyl and carbonyl groups and lead ultimately to the deposition of glucose labeled in all positions. A very small concentration of the 3-carbon symmetrical compound, relative to the concentrations of other intermediates, would suffice to introduce isotope in positions 1, 2, 5, and 6, in the amounts found in the present experiments. It would be unnecessary for the 3-carbon symmetrical compound to lie directly on the metabolic pathway, as long as it was in equilibrium with an actual intermediate, *e.g.* dihydroxyacetone phosphate.

Another possibility deserves mention. When  $C^{14}$ -labeled formate is fed to rats together with glycine, the glucose from the liver glycogen of these animals is labeled predominantly in the 1, 2, 5, and 6 positions.<sup>1</sup> This result may be interpreted by postulating the formation of serine from formate and glycine followed by the transformation of serine to pyruvate.



The pyruvate would be labeled in the methyl group. Passage of this pyruvate through the tricarboxylic acid cycle and via glycolysis to glycogen would result in the distribution of isotope found experimentally. Evidence for reaction (2), serine  $\rightarrow$  pyruvate, has been obtained (4-6). Glycine has been shown to arise from serine by loss of the  $\beta$ -carbon (7). It is the reverse of this process which is postulated above in reaction (1), formate either directly or indirectly furnishing the  $\beta$ -carbon of serine. The formation of serine from glycine has been found in yeast (8), which lends further evidence for the reversibility of the glycine-serine reaction.

If  $CO_2$  directly or via formate may donate to the  $\beta$  position of serine, the presence of isotope in the 1, 2, 5, and 6 positions of the glucose, as found in the present experiments, may be accounted for readily.

Valuable technical assistance by Miss Margaret Cook is gratefully acknowledged.

#### SUMMARY

Glucose isolated from rat liver glycogen after the administration of  $NaHC^{14}O_3$  contains the isotope predominantly in carbons 3 and 4. A trace

<sup>1</sup> Sakami, W., Lifson, N., Lorber, V., and Wood, H. G., unpublished data.

of isotope, however, amounting to about 1 to 2 per cent of that found in the two center carbons, occurs in the other positions in the glucose molecule. Some possible explanations for this finding are advanced.

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# A REACTION OF TRYPTOPHAN WITH METHYL ALCOHOL WHICH DEPENDS UPON THE FORMATION OF FORMALDEHYDE

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(Received for publication, August 22, 1948)

In 1905 Voisenet described a color reaction given by proteins in the presence of an aldehyde and an oxidizing agent (1), and the following year Rosenheim found that the color produced by tryptophan and formaldehyde is spectroscopically identical with that produced with glyoxylic acid (2). Neither of these substances is readily available in a form suitable for the preparation of reproducible reagents, and formaldehyde has the further disadvantage that an excess inhibits formation of the characteristic violet color. We have found that tryptophan reacts with methyl alcohol and sulfuric acid in the presence of copper sulfate to give a similar color. Although this reaction is apparently caused by the formation of formaldehyde, the new reagent may be useful since it is available in purified form and since an excess of the effective aldehyde is avoided by the conditions of the reaction.

## EXPERIMENTAL

The conditions chosen for the reaction are as follows. To 0.2 ml. of a solution of tryptophan are added 3 ml. of absolute methyl alcohol, 0.2 ml. of 5 per cent copper sulfate, and 3 ml. of concentrated sulfuric acid. After standing 2 hours, the absorption of color is measured in a Klett-Summerson colorimeter by using a blank solution containing only the reagents. Fig. 1 presents the results at 540  $m\mu$  in a series of solutions containing varying amounts of tryptophan in the presence of 0.003 to 25 mg. of copper sulfate. Fig. 2 shows the similarity of the absorption curves obtained with an Evelyn colorimeter of the colored products formed with tryptophan by methyl alcohol, glyoxylic acid, and formaldehyde. For the curve with formaldehyde, sodium nitrite was substituted for copper sulfate, since the latter oxidizing agent always produced colored impurities and increased the absorption on each side of the peak.

Neither formaldehyde nor glyoxylic acid can be present as an impurity in the methyl alcohol used, since the reaction can be obtained with aldehyde-free reagent redistilled from sodium bisulfite and from potassium

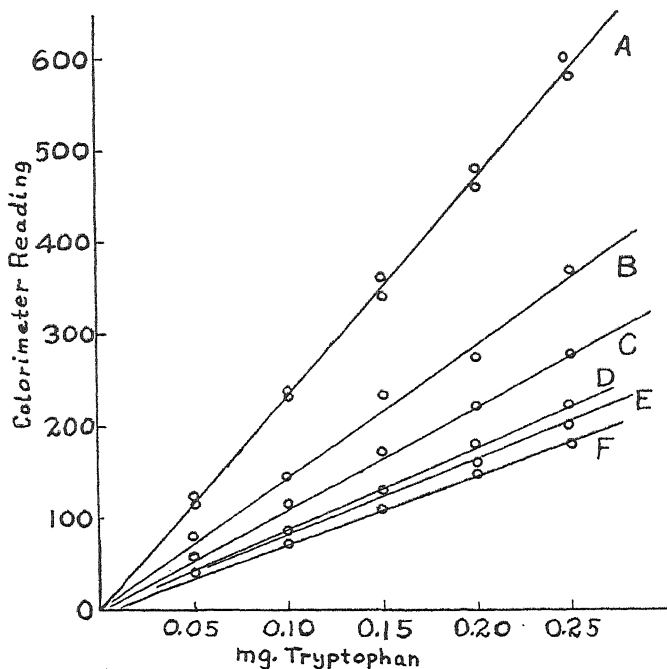


FIG. 1. The effect of varying amounts of copper sulfate. Curve A, 10 and 25 mg. Curve B, 2 mg.; Curve C, 0.4 mg.; Curve D, 0.08 mg.; Curve E, 0.016 mg.; and Curve F, 0.003 mg.

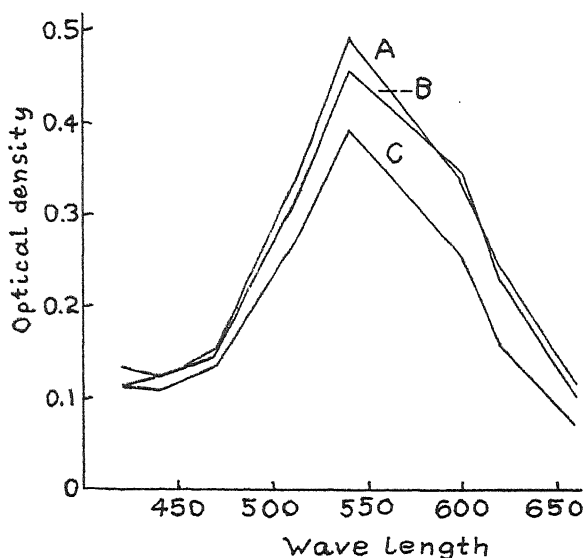


FIG. 2. The absorption curves with methyl alcohol, glyoxylic acid, and formaldehyde. Curve A, glyoxylic acid; Curve B, methyl alcohol; and Curve C, formaldehyde.



hydroxide. The reaction also takes place when potassium methyl sulfate is substituted for methyl alcohol. However, it is probable that formaldehyde is formed by oxidation of methyl alcohol under the conditions of the reaction, since a neutralized mixture of the reagents slowly reduces ammoniacal silver nitrate. A similar faintly positive test for aldehyde is also given by a solution of formaldehyde sufficiently dilute to form a violet color with tryptophan. The formation of formaldehyde with the reagents was also indicated by the color produced with *o*-dianisidine.

The presence of other amino acids causes increased values for tryptophan at 540 and 600  $m\mu$  with methyl alcohol, glyoxylic acid, or formaldehyde, but other amino acids do not affect the results at 420  $m\mu$ . The color measured in the latter region was found to be proportional to the concentration of tryptophan, although the absorption of the characteristic violet color is at a minimum with any of the reagents (Fig. 2). Furthermore, the amount absorbed at 420  $m\mu$  is the same as that of the greenish yellow color formed from tryptophan by methyl alcohol and sulfuric acid in the absence of copper sulfate. We cannot recommend the estimation of tryptophan at 420  $m\mu$ , since the reaction lacks the sensitivity in this region that it has at higher wave-lengths. However, an analysis of crystalline  $\beta$ -lactoglobulin<sup>1</sup> by a method involving this measurement showed 19 mg. of tryptophan per gm. of protein, a value which compares favorably with that reported by Brand (3).

#### SUMMARY

A reaction of tryptophan with methyl alcohol and sulfuric acid in the presence of copper sulfate is described. The absorption curve of the product of this reaction is identical with that obtained with glyoxylic acid and with formaldehyde, and it is concluded that the latter is formed under the conditions of the reaction.

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<sup>1</sup> We are indebted to Professor R. K. Cannan for this preparation.



# URINARY EXCRETION OF AMINO ACIDS BY A NORMAL ADULT RECEIVING DIETS OF VARIED PROTEIN CONTENT\*

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(Received for publication, September 13, 1948)

The urinary excretion of amino acids determined by microbiological assay in normal subjects has been reported from several laboratories (1-4). A moderate variation was found in the quantity of individual amino acids excreted. However, it was observed in this laboratory that, even though the amounts of protein ingested varied from 50 gm. of casein to an estimated 105 gm. of mixed food protein, only 0.4 per cent of the amino nitrogen ingested was lost in the urine in each case (4, 5). These findings suggested that only small changes in the quantity of amino acids excreted might result from large fluctuations in the type and quantity of protein ingested. The experiment reported here was undertaken to determine the effect of wide variations in the protein content of the diet on the excretion of amino acids in the urine of a normal subject under controlled dietary conditions.

## *Materials and Methods*

A healthy 28 year old medical resident in good nutritional status was selected for the study. His diet furnished 3100 calories and was calculated to contain 150, 75, and 0 gm. of protein daily for each of three 8 day periods, respectively. Nitrogen balance and the urinary excretion of the "free" and "combined" forms of the ten "essential" amino acids as well as of  $\alpha$ -amino nitrogen were determined.

The 150 and 75 gm. protein diets contained the foods ordinarily eaten in a well balanced nutritious diet. The nitrogen content was calculated from standard food tables. The protein-free diet supplied only 0.1 gm. of nitrogen daily as determined by macro-Kjeldahl analysis. Its composition and ability to maintain nitrogen balance and constant weight with the addition of 85 gm. of purified casein (Labco) as the sole source of nitrogen have previously been described (6).

\* The expenses of this investigation were defrayed in part by a grant from Merck and Company, Inc., to Harvard University.

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The 24 hour urine samples were preserved with 10 cc. of a 5:1 mixture of glacial acetic acid and toluene. Suitable aliquots were stored at  $-20^{\circ}$  for microbiological assay of individual amino acids, and at  $4^{\circ}$  for determination of total nitrogen and  $\alpha$ -amino nitrogen. The "free" forms of the eight amino acids "essential" for man (7) and of arginine and histidine were determined by the microbiological assay method of Stokes *et al.* (8). The "free"  $\alpha$ -amino nitrogen was determined by the gasometric-ninhydrin method as described by Van Slyke, MacFadyen, and Hamilton (9). After hydrolysis of the urine with an equal volume of concentrated hydrochloric acid at  $105^{\circ}$  for 24 hours, the analyses were repeated for the  $\alpha$ -amino nitrogen and the "total" of the individual amino acids, except tryptophan. Since tryptophan is destroyed upon acid hydrolysis, the "total" form of this amino acid was determined microbiologically after alkaline hydrolysis of the urine with an equal volume of 10 N sodium hydroxide at  $105^{\circ}$  for 24 hours. The difference between the "free" and "total" values was considered to represent the quantity of amino acids present in "combined" form. This will be referred to as the quantity of "peptides" present, although amino acids with other than the peptide linkage may be released upon hydrolysis.

Separate analyses were made on pooled urines of the first 4 days, the next 2 days and the last 2 days for each of the three 8 day periods. Since no significant changes occurred in the excretion of individual amino acids or of  $\alpha$ -amino nitrogen during each dietary régime, only the average of each 8 day period will be reported.

### Results

The subject maintained a constant weight throughout the 24 day study period. The nitrogen balance averaged  $+5.5$  gm.,  $-0.1$  gm., and  $-3.8$  gm. daily for the 150, 75, and 0 gm. protein diet periods, respectively. There was a moderate decrease in the urine total nitrogen by the 5th day of the protein-free period and thereafter it averaged 2.9 gm. per 24 hours.

The values for the ten amino acids essential for rats and for the  $\alpha$ -amino nitrogen excreted in the urine during the three dietary periods are presented in Table I and plotted in Fig. 1. The free form and the combined (peptide) form of individual amino acids and of  $\alpha$ -amino nitrogen decreased as the protein intake in the diet was reduced. However, large decreases in the quantity of protein ingested resulted only in moderate decreases in the urinary excretion of amino acids and of peptides compared to their excretion on the protein-free diet.

The ratio of free amino acids to peptides in the urine remained essentially constant throughout each dietary régime, whether determined for individual amino acids or for  $\alpha$ -amino nitrogen. As the protein intake was

# RELATION BETWEEN PROTEIN INTAKE AND URINARY AMINO ACID EXCRETION

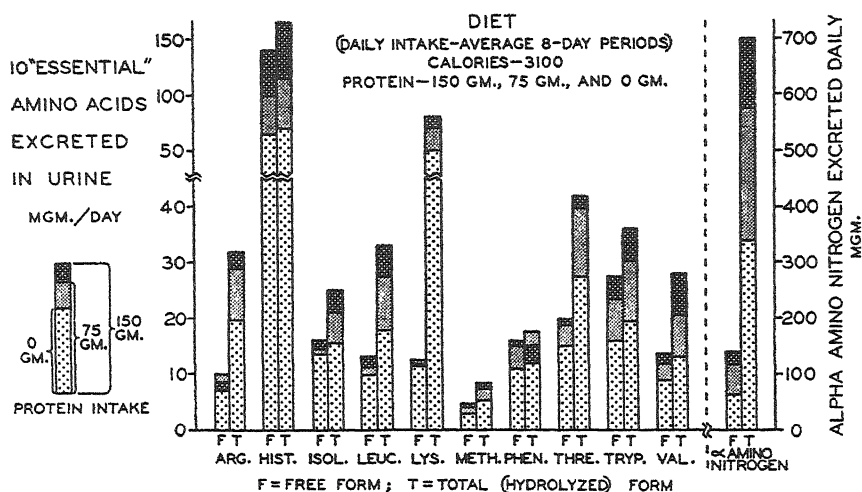


FIG. 1

TABLE I

*Relation between Protein Intake and Urinary Amino Acid Excretion by Normal Adult*

The results are averages for 8 days, in mg. per 24 hours; the totals are given in parentheses.

		150 gm. protein	75 gm. protein	0 gm. protein
Arginine	Free	8.6	10.1	7.0
	Bound	23.3 (31.9)	18.6 (28.7)	12.7 (19.7)
Histidine	Free	14.0	99.3	65.2
	Bound	25.0 (165)	16.7 (116)	4.9 (70.1)
Isoleucine	Free	16.1	14.4	13.5
	Bound	9.0 (25.1)	6.8 (21.2)	1.9 (15.4)
Leucine	Free	12.9	11.3	9.7
	Bound	20.3 (33.2)	16.2 (27.5)	8.2 (17.9)
Lysine	Free	11.4	12.5	11.8
	Bound	68.9 (80.3)	57.8 (70.3)	37.9 (49.7)
Methionine	Free	4.7	4.0	2.8
	Bound	3.8 (8.5)	3.6 (7.6)	2.5 (5.3)
Phenylalanine	Free	15.9	15.1	10.9
	Bound	0.0 (15.9)	2.7 (17.8)	0.8 (11.7)
Threonine	Free	20.1	18.9	14.8
	Bound	21.7 (41.8)	20.9 (39.8)	12.6 (27.4)
Tryptophan	Free	27.4	23.4	15.8
	Bound	8.8 (36.2)	6.9 (30.3)	3.6 (19.4)
Valine	Free	13.7	11.9	9.0
	Bound	14.4 (28.1)	8.8 (20.7)	3.9 (12.9)
α-Amino N	Free	140	118	63.0
	Bound	558 (698)	457 (575)	277 (340)

reduced, therefore, a proportionate decrease occurred in the excretion of amino acids in the free and combined forms. The excretion of combined  $\alpha$ -amino nitrogen (peptides) continued throughout the protein starvation régime and represented about 4 times the excretion of free  $\alpha$ -amino nitrogen, a ratio of peptides to free amino acids identical to that excreted by this subject while receiving the diets containing 75 and 150 gm. of protein. No significant decrease occurred in the excretion either of free amino acids or of peptides throughout the 8 day protein starvation period.

The relation between the total amino acids administered during each dietary régime and their urinary excretion is shown in Table II. From 0.4

TABLE II

*Relation between Protein Intake and Quantity of Amino Acids (Essential and Non-Essential) Excreted in Urine in Free Form*

Protein intake	Total amino acids		Total 10 "essential" amino acids excreted†	10 "essential" amino acids excreted	Total amino acids excreted‡
	Administered	Excreted*			
gm.	mg. per 24 hrs.	mg. per 24 hrs.	mg. per 24 hrs.	per cent total excreted	per cent total administered
150	150,000	1094	271	24.8	0.4
75	75,000	919	221	24.0	0.6
0	0	494	161	32.6	

\* Calculated from the free  $\alpha$ -amino nitrogen excretion values, Table I, assuming  $\alpha$ -amino nitrogen to be 80 per cent of total nitrogen and total nitrogen to be 16 per cent of amino acids.

† Total of the free forms of the ten "essential" amino acids excreted, Table I.

‡ Per cent of administered amino acids excreted in excess of that excreted on the protein-free diet.

to 0.6 per cent of the protein ingested was excreted in the urine as free amino acids in excess of that excreted on the protein-free diet. The ten "essential" amino acids contributed approximately one-fourth of the total amino acids excreted.

#### DISCUSSION

It was observed in this study that the urinary excretion of the free and combined forms of individual amino acids and of  $\alpha$ -amino nitrogen continued throughout an 8 day period in which a normal subject received adequate calories but no protein. The urinary excretion of free amino acids and peptides which occurred while no protein was fed must be considered endogenous in origin.

When protein was fed an increase in the excretion of amino acids and peptides occurred, but large increases in the quantity of protein ingested

resulted only in small increases in the quantity of amino acids excreted (Table I and Fig. 1). Thus a large portion of the amino acids in the urine of subjects eating *ad libitum* may be considered to be of "endogenous" and only a small portion of "exogenous" origin.

The "exogenous" excretion of the free forms of individual amino acids and of  $\alpha$ -amino nitrogen probably results from spillage in the urine of amino acids coincident with their postprandial increase in the plasma. A similar elevation of plasma peptides could account for the greater urinary excretion of combined amino acids when protein rather than a protein-free diet is ingested. However, Christensen and coworkers were unable to demonstrate an elevation of plasma peptide levels after oral protein feeding (10). Nevertheless, early investigations strongly suggest that partial breakdown products of protein (and even minute amounts of intact proteins) may be absorbed into the circulation (11). Silber, Howe, and Porter have recently noted that the ingestion of partial protein hydrolysates resulted in a greater peptiduria than when intact protein was fed (12). These conflicting reports leave unclear the mechanism responsible for the peptiduria which follows protein feeding. Likewise, it is not possible to explain why the proportion of free amino acids and peptides excreted in the urine remained constant despite marked changes in the protein content of the diet.

Peptides are apparently less efficiently retained by the kidney than are free amino acids. Although peptides are present in much lower concentration in the plasma than are free amino acids (10), they are excreted in the urine in greater concentration. Further, conjugated amino acids are lost to a greater extent in the urine than are free amino acids following intravenous infusion (13, 14).

It is often desirable to ascertain the amount of administered amino acids lost in the urine. To accomplish this, it is obligatory to determine the quantity of amino acids excreted during a period when no protein is ingested. The "excess" amino acids excreted while protein is being administered (whole protein orally or hydrolyzed protein intravenously) may then be presumed to arise from the administered protein *per se*. The "endogenous" urinary amino acid excretion can be obtained with certainty only after a subject has received a diet adequate in calories but free of protein for several days. However, it might be practicable to collect a fasting urine specimen to obtain an approximate value for the minimum loss of amino acids. The error incurred by the assumption that the amino acids in a fasting morning specimen are largely "endogenous" would be far less than in assuming, as others have (1), that all the amino acids arise from the protein ingested.

Variations in the type and quantity of protein eaten result in the urinary excretion of free  $\alpha$ -amino nitrogen of only approximately 0.5 per cent of that given (Table II) (4, 5). The quantity of individual amino acids excreted was only slightly greater when a high protein or a moderate protein rather than a protein-free diet was eaten (Table I and Fig. 1). Woodson *et al.*

TABLE III

*Comparison of Urinary Amino Acid Excretion by Adults Receiving Diets of Varied Type and Quantity of Protein (Mg. per 24 Hours)*

		Normal diet (1)	Normal diet (2)	Normal diet (3)	Normal diet (4)	Casein (5)	K ration (6)	Egg (7)	Boiled soy bean (8)	Auto- claved soy bean (9)
Arginine	Free	21.3	9.4	20.0	9.5	14.0		4.4	5.1	8.1
	Total	23.7	30.3	35.6		39.3	41.5			
Histidine	Free	188	120	135	163	159		74.6	73.2	69.3
	Total	203	141	188		200	252			
Isoleucine	Free	5.9	15.3	3.3	13.8	5.4		5.0	4.5	4.5
	Total	20.3	23.2	19.3		17.7	19.5			
Leucine	Free	9.6	12.1	10.6	7.2	16.2		0	0	13.0
	Total	21.2	30.4	31.2		32.2	39.1			
Lysine	Free	33.6	12.0	19.0	46.8	39.3		1.5	3.0	1.5
	Total	73.2	75.3	83.1		120	75.7			
Methionine	Free	7.8	4.4	2.7	4.4	4.9		2.4	2.5	4.3
	Total	8.6	8.1	11.9		9.1	22.0			
Phenylalanine	Free	16.4	15.5	9.8	14.0	24.0		4.9	7.1	3.2
	Total	23.3	16.5	32.6			31.8			
Threonine	Free	24.4	19.5	23.5	20.9	24.0		24.9	19.7	22.0
	Total	53.8	40.8	57.9		39.6	66.0			
Tryptophan	Free	24.6	25.4	23.6	12.7	12.5		7.5	7.5	8.4
	Total	41.4	33.3							
Valine	Free	4.5	12.8	5.0	7.4	9.5		7.3	4.1	9.2
	Total	19.8	24.4	29.7		29.0	36.6			

Column 1, Woodson *et al.* (3); Column 2, present study, averaged value of 150 and 75 gm. protein periods; Columns 3, 6, Dunn *et al.* (2); Column 4, Eckhardt and Davidson (4); Column 5, Eckhardt, Murphy, and Davidson (unpublished data), averaged value of normal subject given 85 gm. of Labco casein daily for 6 days; Columns 7, 8, 9, Steele *et al.* (1).

also noted that "the amino acids excreted by the different subjects show no significant variations in amount, although there is a wide variation in the type of 'normal' diets, caloric intake, urine volume, and total nitrogen excretion" (3). Likewise, Steele *et al.* observed that "the amounts of any one amino acid excreted by human subjects were essentially the same whether eggs or boiled soy beans were fed, in spite of differences in the amino acid content of the two diets" (1). Therefore, the quantity of



amino acids excreted in the urine by human subjects would not be expected to vary greatly, even though the diets were quite different.

However, as noted in Table III, a variation exists in the urinary excretion of the free amino acids reported from different laboratories (1-4), while the variation is less for the total amino acids assayed after hydrolysis. There are several explanations for these divergent values, as previously pointed out by Dunn *et al.* (2). In urine preserved with acid, as was done by Steele *et al.* (1), and in the present study, hydrolysis of a portion of the combined amino acids would result in falsely high free amino acid values. This may explain, for example, the greater excretion of the free form of isoleucine (both in absolute amount and in per cent of the total) observed in this study than that observed by others (Table III, Column 2 contrasted to Columns 1, 3, and 5). The microbiological assay of free amino acids is technically more difficult than that of the total (hydrolyzed) form, since untreated urine contains a lower concentration of free amino acids as well as a greater concentration of inhibitory substances (chiefly urea) than hydrolyzed urine. In fact, Dunn *et al.* were able to detect leucine, isoleucine, and lysine "consistently and satisfactorily only in urines which have been hydrolyzed with acid" (2). Finally, slightly different amino acid values (both free and total) might be expected to result from differences in the microorganism used and in the assay techniques employed in the various laboratories. Because large fluctuations in the type and quantity of protein eaten result in only small changes in the quantity of amino acids excreted, the variations observed in the free and total amino acid excretion tabulated in Table III may be explained largely by differences in urine preservation and assay techniques rather than by differences in the variety and quantity of protein ingested.

#### SUMMARY AND CONCLUSIONS

A normal subject was given a diet adequate in calories and calculated to contain 150, 75, and 0 gm. of protein daily for each of three 8 day periods, respectively. The nitrogen balance averaged +5.5, -0.1, and -3.8 gm. daily for these periods. The subject maintained a constant weight throughout the study.

Approximately 0.5 per cent of the  $\alpha$ -amino nitrogen given was excreted in the urine. The free and the combined (peptide) forms of individual amino acids and of  $\alpha$ -amino nitrogen in the urine decreased as the protein intake in the diet was reduced. However, large decreases in the quantity of protein ingested resulted only in moderate decreases in the urinary excretion of amino acids and peptides compared to their excretion on the protein-free diet. No significant decrease occurred in the excretion of either free amino acids or peptides during the 8 day protein starvation period.

The excretion of free  $\alpha$ -amino nitrogen was about one-fourth that of the combined  $\alpha$ -amino nitrogen (peptides). The ratio of free amino acids to peptides in the urine remained essentially constant throughout each dietary régime. As the protein intake was reduced, the ratio was maintained so that a proportionate decrease occurred in the excretion of amino acids in both forms.

There is a greater concentration of peptides in the urine than in the plasma. In addition, there is a greater urinary excretion of peptides than of free amino acids following their intravenous infusion. These facts suggest that combined amino acids are less efficiently retained by the kidney than are free amino acids.

The urinary excretion of free amino acids and peptides which occurred while no protein was fed must be considered "endogenous" in origin. Since this minimum excretion was increased only slightly by the ingestion of protein, a large portion of the amino acids in the urine of subjects eating *ad libitum* is of "endogenous" and a small portion of "exogenous" origin.

The moderate variation observed in the free and total amino acid excretion reported from different laboratories may be explained by differences in urine preservation and assay technique rather than by differences in the protein ingested, since large fluctuations in the type and quantity of protein eaten result in only small changes in the quantity of amino acids excreted.

The authors wish to thank Miss Elaine Hirshberg and Miss Virginia Garrison for their technical assistance, and Mrs. Jean Harrison for preparing and calculating the diets used.

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# MICROBIOLOGICAL DETERMINATION OF LEUCINE IN PROTEINS AND FOODS

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(Received for publication, October 6, 1948)

The assay of leucine by microorganisms offers an easy and accurate method compared to its determination by chemical means. A wide se-

TABLE I  
*Recovery of Leucine Added to Protein Hydrolysates*

Protein hydrolysate	Leucine				
	In hydrolysate*	Added	Total	Found	Recovery
	$\gamma$	$\gamma$	$\gamma$	$\gamma$	per cent
Barley, pearled.....	4.00	20.00	24.00	23.80	99
	8.00	20.00	28.00	28.00	100
	12.00	20.00	32.00	32.00	100
	16.00	20.00	36.00	36.00	100
Conarachin.....	12.20	5.00	17.20	17.40	101
	12.20	10.00	22.20	21.00	94
	12.20	15.00	27.20	27.20	100
Corn, whole, yellow.....	16.80	20.00	36.80	36.80	100
	16.80	40.00	56.80	57.50	101
	16.80	60.00	76.80	75.00	97
Gelatin (Bacto).....	2.85	5.00	7.85	7.90	101
	5.70	5.00	10.70	10.40	97
	8.55	5.00	13.55	13.00	96
	11.40	5.00	16.40	16.20	99
Zein.....	37.50	5.00	42.50	42.30	99
	37.50	10.00	47.50	48.00	101
	37.50	15.00	52.50	53.50	102
	37.50	20.00	57.50	59.20	103

\* Not corrected for moisture and ash.

lection of media and test organisms for the determination of this amino acid is offered in the literature (1-21).

A satisfactory standard curve was developed in this laboratory with *Leuconostoc mesenteroides* with the medium reported for methionine (22) and modified as used for lysine (23) and threonine (24).

## EXPERIMENTAL

*Leuconostoc mesenteroides* P-60<sup>1</sup> was employed in the assays described.  
*Basal Medium*—The basal medium was the same as that described in a

TABLE II

*Leucine Content of Some Proteins and Foods Determined at Different Assay Levels\**

Protein assay level	Leucine found									
	Zein		Wheat bran globulin		Soy bean flour		Whole wheat		Whole rye	
$\gamma$	$\gamma$	per cent	$\gamma$	per cent	$\gamma$	per cent	$\gamma$	per cent	$\gamma$	per cent
100	20.20	20.20	6.10	6.10						
200	40.70	20.35	12.00	6.00	7.50	3.75				
300	60.70	20.23	17.70	5.90						
400	80.80	20.20	24.00	6.00	14.80	3.70				
500							5.00	1.00		
600					22.20	3.70				
800					29.20	3.65				
1000							10.00	1.00	7.40	0.74
1500							15.00	1.00		
2000							20.00	1.00	14.80	0.74
3000									21.80	0.73
4000									29.20	0.73
Average.....		20.24		6.00		3.70		1.00		0.74

\* Not corrected for moisture and ash.

TABLE III

*Reproducibility (Per Cent) of Leucine Content When Determined by Separate Assays\**

Material	Assay 1	Assay 2	Average
Casein.....	8.55	8.54	8.55
Corn, whole, yellow.....	1.68	1.65	1.67
Oatmeal.....	1.19	1.19	1.19
Ox muscle.....	8.53	8.60	8.56
Peanut flour.....	3.84	3.80	3.82
Rice, white.....	0.61	0.60	0.61
Yeast, dried, brewers'.....	2.86	2.91	2.88
Zein.....	20.24	20.00	20.12

\* Not corrected for moisture and ash.

previous paper for methionine (22) and modified in other papers (23, 24).

*Assay Procedure*—The procedures followed for the cultures, inoculum,

<sup>1</sup> Obtained from the American Type Culture Collection, Georgetown University School of Medicine, Washington, D. C.

and preparation of samples were identical with those described in other papers (22, 24).

TABLE IV  
*Leucine Content of Some Proteins and Foods*  
Percentages calculated for ash- and moisture-free material.

Material	N	Leucine	Values from literature
	<i>per cent</i>	<i>per cent</i>	
Arachin.....	18.80	7.61	
Casein.....	16.07	9.22	9.8 (8), 8.1 (10), 9.6 (11), 9.8 (13), 9.9 (14), 9.5 (15), 10.8 (17), 9.9 (18), 11.1 (26), 9.1 (27), 9.4 (28), 14.4 (29), 12.1 (30)
Coconut globulin.....	17.42	7.18	5.96 (31)
Conarachin.....	18.20	6.61	
Cottonseed globulin.....	18.00	7.06	6.8 (13), 8.4 (32)
Edestin.....	18.55	7.50	5.5 (10), 7.4 (13), 7.0 (30), 7.4 (33)
Gelatin (Bacto).....	18.82	3.30	3.6 (13), 3.5 (14), 3.2 (15), 3.2 (17), 2.7 (26), 3.3 (28), 3.5 (34)
Glycinin.....	17.80	8.07	8.4 (13)
Lactalbumin.....	15.89	12.90	10.8 (15), 10.8 (27)
Ovalbumin (crystalline)....	15.98	9.43	9.6 (13), 9.2 (14), 9.1 (34)
Ox muscle.....	16.00	9.37	7.7 (15)
Peanut, total globulins.....	18.01	7.45	8.1 (13)
Phaseolin (navy bean).....	16.07	10.50	
Wheat bran globulin.....	17.76	6.35	
Zein.....	16.00	21.10	15.4 (13), 23.7 (30)
Barley, pearled.....	1.86	0.89	0.64 (17)
Brazil nut meal.....	9.03	4.09	
Corn germ, defatted.....	3.93	1.83	1.65 (26), 1.89 (35)
Corn, whole, yellow.....	2.22	1.99	2.07 (17), 2.98 (30)
Cottonseed flour.....	10.86	3.81	3.24 (5), 3.56 (17), 3.24 (30), 3.88 (35)
Egg, whole, dried.....	8.11	6.37	4.66 (36)
Milk, dry, skim.....	6.57	4.24	4.07 (14), 3.78 (17), 4.35 (18), 4.64 (26), 3.96 (37), 3.57 (38)
Oatmeal.....	2.73	1.32	1.12 (17), 1.37 (30)
Peanut flour.....	10.15	4.25	3.49 (5), 3.49 (30), 4.25 (35)
Peas, black eyed.....	4.15	2.06	
Rice, white.....	1.26	0.68	0.61 (30)
Rye, whole.....	1.98	0.83	0.77 (14)
Soy bean flour.....	8.85	4.16	3.65 (5), 4.09 (14), 4.36 (17), 4.10 (26), 3.65 (30), 4.20 (35), 4.19 (39)
Wheat germ, defatted.....	6.50	2.44	2.03 (26), 3.01 (30), 2.68 (35)
“ whole.....	3.07	1.13	1.11 (5), 1.30 (14), 1.18 (17), 1.11 (30)
Yeast, dried, brewers'.....	7.71	3.25	3.42 (14), 2.92 (26), 3.56 (40)

*Preparation of Leucine Standards*—L-Leucine was used in preparing the standard solutions. The titration values on the standard curve (Fig. 1)

were not altered by the addition to the medium of 1.2 mg. of any of the other nineteen amino acids.

Recovery of leucine added to hydrolysates of barley, conarachin, corn, gelatin, and zein gave results well within the experimental error for this type of assay (Table I).

Table II shows values found for zein and several foods at different assay levels. Data on the reproducibility of values found for a number of materials when determined by separate assays are given in Table III.

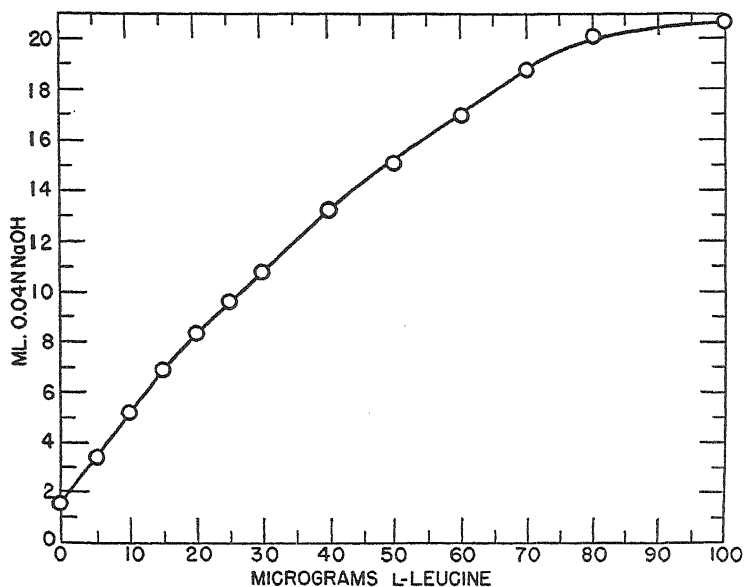


FIG. 1. Standard titration curve

The results (Table IV) found for the proteins and foods<sup>2</sup> agree quite well with other values obtained by the use of microbiological methods. The results on several of the proteins are very close to those obtained by the use of *Neurospora* and by the solubility method, but, in general, are higher than those obtained by chemical assay.

#### SUMMARY

A microbiological method is described for the determination of leucine in proteins and foods with *Leuconostoc mesenteroides* P-60. The results of assays on thirty-one proteins and foods agree closely with those obtained on the same materials by other microbiological methods.

<sup>2</sup> The sources and preparations of the samples assayed are given in a previous publication on the determination of methionine (25).



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## A SITE OF ACTION OF STREPTOMYCIN

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(Received for publication, July 24, 1948)

Various reports (1-5) have demonstrated that streptomycin inhibits the metabolic activities of the non-proliferating cells of susceptible strains of bacteria. This paper is an attempt to trace the mode of action of streptomycin with the object of determining what reactions vital to the cell are interfered with. Study of the mode of action of the antibiotics of therapeutic value should eventually provide an explanation for their ability to enter the animal body and kill susceptible bacteria without killing the tissue cells. The first step toward this goal is to determine which reactions of the bacterium are susceptible to streptomycin inhibition at concentrations of this agent which seem to be effective in practice.

In order to investigate this problem, we began with the observations of Geiger (3), who demonstrated that, with a particular strain of *Escherichia coli*, there was a marked stimulation of serine oxidation by the previous oxidation of fumarate. When streptomycin was present (at levels of 5 to 20  $\gamma$  per ml.) during the oxidation of fumarate, this stimulation was prevented. Geiger concluded that streptomycin prevents the *action* of some intermediate, formed during the oxidation of fumarate and a variety of other carbohydrate substances, which is necessary for the oxidation of serine. One could also interpret the data reported by Geiger as indicating that it was the *formation* of the intermediate which was interfered with by streptomycin. Using these observations as a starting point, we have been able to trace the mode of action of streptomycin somewhat further.

### Methods

The Gratia strain of *Escherichia coli*<sup>1</sup> was grown in 1 per cent Difco peptone, 1 per cent Difco yeast extract, 0.5 per cent  $K_2HPO_4$  (Medium A) at 30° or 37° (as specified) for 16 to 18 hours. The cells were harvested by centrifugation, washed three times by resuspension in one-tenth the growth volume of distilled water, and suspended in distilled water to give a cell concentration of from 0.5 to 1.25 mg. of bacterial nitrogen per ml. Such suspensions retained the activities to be described for 3 to 5 days at refrigerator temperatures, but thereafter the activity was gradually lost. As noted by Geiger (3), cocarboxylase was effective in restoring the lost activity for

<sup>1</sup> Sensitive in culture to 9  $\gamma$  per ml. of streptomycin. We are indebted to Mr. Otto Graessle for these determinations.

a short period, but with our strain other deficiencies soon developed. The properties of the living cell suspensions vary somewhat with the growth medium (if peptone is replaced in equal amounts by N-z-amine (Medium B) or if glucose is supplied during growth), the growth temperature, the concentration of cells, the conditions under which the measurements are made, and doubtless other factors. These variations are of a quantitative rather than a qualitative character, and are reflected in the amounts of gas exchange observed or the degree of streptomycin inhibition. Since our object was to trace the mode of action of streptomycin rather than to study the influence of various factors on streptomycin activity, we attempted to select, as the work progressed, those conditions most conducive to demonstrating the "streptomycin effect."

Streptomycin was used as the commercial (Merck) calcium chloride complex at a level of 10  $\gamma$  of the free base per ml. (except where otherwise specified). Streptomycin hydrochloride was equally effective, but the calcium chloride complex inactivated by heat and acid or alkali was not effective. Total keto acids were determined by the method of Friedemann and Haugen (6).  $\alpha$ -Ketobutyrate was determined by the total keto acid method, standardized against its 2,4-dinitrophenylhydrazone.<sup>2</sup> While, in general, the specificity of this procedure could be questioned, in this particular case it is reasonably valid, since the 2,4-dinitrophenylhydrazone of  $\alpha$ -ketobutyrate is much less soluble than those of most other keto acids and actually precipitates in the initial stages of the total keto acid procedure with samples containing as little as 10  $\gamma$  of  $\alpha$ -ketobutyrate. The  $\alpha$ -ketobutyrate used in a few experiments was prepared from the N-benzoylaminocrotonic acid<sup>3</sup> after the method of Carter *et al.* (7). Ammonia was determined by nesslerization (8) after removing the acid-killed cells by centrifugation. Acetyl phosphate was estimated by the method of Lipmann and Tuttle (9), standardized against succinic anhydride. Standard Warburg techniques (8) were used throughout. All respiration measurements were made at 37° at pH 7.

#### *Studies with Threonine*

The studies were first directed towards the oxidation of amino acids as reported by Geiger (3). It was soon found that neither the D- nor L-amino acid oxidases from either bacteria or animal tissues were inhibited by streptomycin, and that the Gratia chain of *Escherichia coli* was capable of attacking only aspartate, serine, or threonine at a rapid rate. While

<sup>2</sup> Obtained through the generosity of Dr. D. B. Sprinson, Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York.

<sup>3</sup> We are indebted to Dr. E. E. Howe of the Research Laboratories of Merck and Company, Inc., for this material.

some study was devoted to serine and the results of Geiger were confirmed in principle, our strain did not show the gradually increasing rate of serine oxidation found by Geiger.

The threonine system was more readily approachable. As with serine, the oxidation of threonine could be markedly increased by the previous

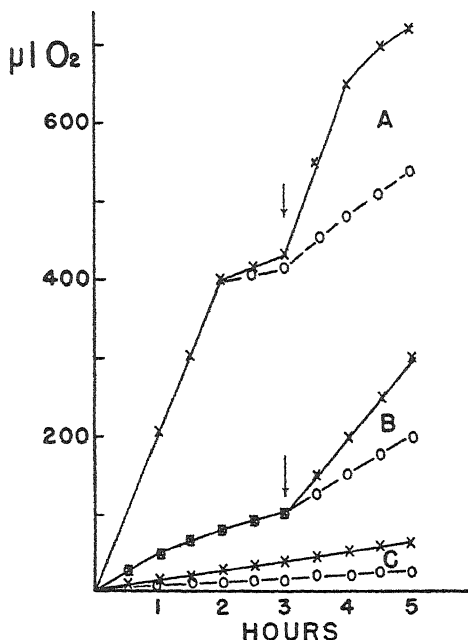


FIG. 1. Influence of fumarate oxidation and streptomycin upon the oxidation of threonine. Curves A, 10 micromoles of fumarate added at zero time, 10 micromoles of DL-threonine added at 3 hours; Curves B, threonine at zero time, fumarate at 3 hours. The broken lines represent treatments containing 20  $\gamma$  per ml. of streptomycin. The cups contain 0.25 mg. of bacterial nitrogen, 0.016 M phosphate buffer, pH 7, substrates, and water to make 3 ml.; gas phase air; temperature 37°.

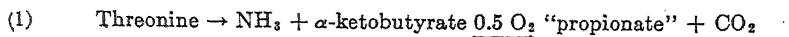
oxidation of fumarate. If streptomycin was present during the oxidation of fumarate, little increased rate of threonine oxidation was observed.

A typical experiment is illustrated in Fig. 1. First it may be noted from Curves C that a small amount of oxygen is consumed in the absence of added substrate, which may or may not show a streptomycin inhibition as in this case. For simplicity in presentation, the curves in this and subsequent figures have been corrected for the respiration in the absence of substrate. From Curves B it will be noted that there is a relatively slow oxidation of threonine. Fumarate is oxidized more slowly if it is added 3

hours after threonine than if it is added at zero time, and the oxidation of fumarate added after threonine is inhibited by streptomycin. From Curves A the initial addition of 10 micromoles of fumarate causes a rapid oxygen uptake for 2 hours, which is not altered in rate by the presence of streptomycin. After 2 hours the rate slows and this rate (between the 2nd and 3rd hours) is, in this case, slightly inhibited by streptomycin. The inhibition over this period is variable and is greatly dependent upon the salt concentration in which the reaction is run. At the 3rd hour, 10 micromoles of DL-threonine were added. It is evident that the oxidation of fumarate has promoted the subsequent oxidation of threonine and that streptomycin, when present during the fumarate oxidation, has prevented this phenomenon.

It first appeared that, if one could determine by what pathway the threonine was oxidized, one could determine the mode of action of streptomycin. This strain of *Escherichia coli*, grown under the conditions described, possesses a threonine deaminase which, as has been shown by Chargaff and Sprinson (10), produces ammonia and  $\alpha$ -ketobutyric acid. In the case of data comparable to those given in Fig. 1, per micromole of threonine added, we found 0.9 to 1.1 micromoles of ammonia, with or without previous fumarate oxidation or in the presence or absence of streptomycin. Further D-, L-, or DL-threonine gave comparable results, characteristic of threonine deaminase (10). The other product of deamination is oxidized by most suspensions, but if oxidation is stopped before the reaction has used 0.5 mole of  $O_2$  per mole of threonine, keto acid accumulation with the characteristics of  $\alpha$ -ketobutyrate can be detected. We have, therefore, concluded that the first step in the oxidation of threonine is deamination to  $\alpha$ -ketobutyrate and ammonia. This is independent of previous fumarate oxidation in the presence or absence of streptomycin; hence the streptomycin effect noted is concerned with the oxidation of  $\alpha$ -ketobutyrate. The most direct way of establishing this fact would be to substitute  $\alpha$ -ketobutyrate for threonine, but unfortunately added  $\alpha$ -ketobutyrate is but slowly oxidized (with or without the simultaneous addition of ammonia), even after intensive fumarate oxidation, possibly because at pH 7 it does not readily penetrate the cell.

The data of Fig. 2 summarize the effects of previous oxidation of fumarate and the presence or absence of streptomycin upon the oxidation of threonine. Threonine added to cells which have not previously oxidized fumarate take up close to 0.5 mole of  $O_2$  per mole of threonine and simultaneously release close to 1.0 mole of  $CO_2$  (Figs. 2, A, 2, B). These data are in accordance with Reaction 1 which is similar to that reported by Long



and Peters (11) for the oxidation of  $\alpha$ -ketobutyrate by brain tissue. Reaction 1, as is evident from Fig. 2, is not influenced by streptomycin. Further, since the same results are obtained whether the threonine is added at zero time or after 3 hours of endogenous respiration, the respiration in the absence of added substrate under these conditions does not either increase the toxicity of streptomycin or generate the ability to oxidize threonine further. Occasional suspensions, as might be expected, do

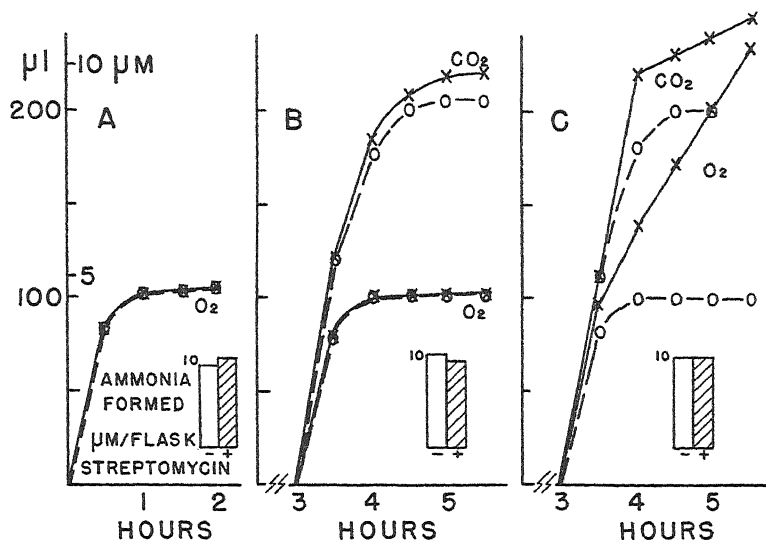


FIG. 2. Oxidation of threonine. In A, 10 micromoles of threonine were added at zero time; in B, added after 3 hours respiration without substrate; in C, added after 3 hours respiration on 10 micromoles of fumarate. 1 mg. of bacterial nitrogen per cup in 0.003 M phosphate, pH 7. The broken lines represent treatments containing 10  $\gamma$  of streptomycin per ml.

show increased respiration with threonine after 3 hours of endogenous respiration. This effect we have attributed to the occasional presence of oxidizable substrates in the cell. In the presence of streptomycin the addition of threonine after 3 hours of fumarate oxidation leads to the same result (0.5 mole of  $\text{O}_2$ , 1.0 mole of  $\text{CO}_2$  per mole of threonine) as does the addition of threonine at zero time or after 3 hours of endogenous respiration. However, if streptomycin is not present, the oxidation of fumarate permits the oxidation of threonine to proceed much further (Fig. 2, C). While the reaction written in Reaction 1 probably represents the course of threonine oxidation when added without previous fumarate oxidation (or with fumarate oxidation in the presence of streptomycin), it does not

necessarily represent the first stage of the reactions when fumarate has been oxidized. Here, as will be noted from Fig. 2, 1 mole of  $\text{CO}_2$  and 1 mole of ammonia are formed; yet the product of fumarate oxidation may react with the  $\alpha$ -ketobutyrate before the latter is oxidized. Thus, the oxidation of added propionate may not be stimulated by previous fumarate oxidation. This is indeed the case; added propionate is virtually inert, either because of the reasons outlined above, or because it does not enter the cell.

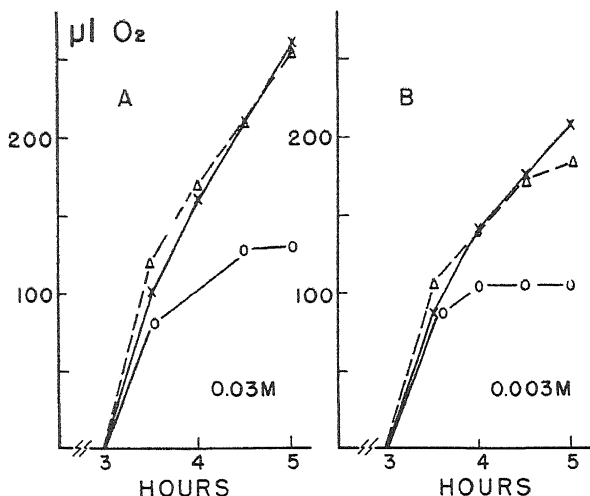


FIG. 3. Oxidation of threonine after fumarate oxidation. 1.0 mg. of bacterial nitrogen per cup. 10 micromoles of fumarate oxidized for 3 hours, then 10 micromoles of threonine added. A, 0.03 M phosphate buffer, pH 7; B, 0.003 M phosphate buffer. X, no streptomycin; O, 10  $\gamma$  per ml. of streptomycin present during fumarate oxidation;  $\Delta$ , 10  $\gamma$  per ml. of streptomycin added at 3 hours with threonine.

These results lead to a conclusion similar to that reached by Geiger (3); i.e., streptomycin prevents the formation or activity of some substance derived from the oxidation of fumarate which is necessary for the oxidation of threonine (or its deamination product,  $\alpha$ -ketobutyrate). It was, therefore, desirable to attempt to distinguish between the possibility that streptomycin prevented the formation of this substance and the possibility that it prevented its action. If fumarate were oxidized in the absence of streptomycin, the substance should be formed. If its action were prevented by streptomycin, then the addition of streptomycin with the threonine should be effective, whereas if its formation were prevented by streptomycin, the latter should be active only when present during fumarate oxidation. The data of Fig. 3 indicate that to be really effective the streptomycin must be



present during fumarate oxidation. This agent therefore apparently acts by preventing the formation of the hypothetical intermediate rather than by interfering with its action once it is formed.

### *Studies with Organic Acids*

In efforts to identify the substance which is formed during the oxidation of fumarate and whose formation is inhibited by streptomycin, experiments were done at a low phosphate level, at which effects of streptomycin upon

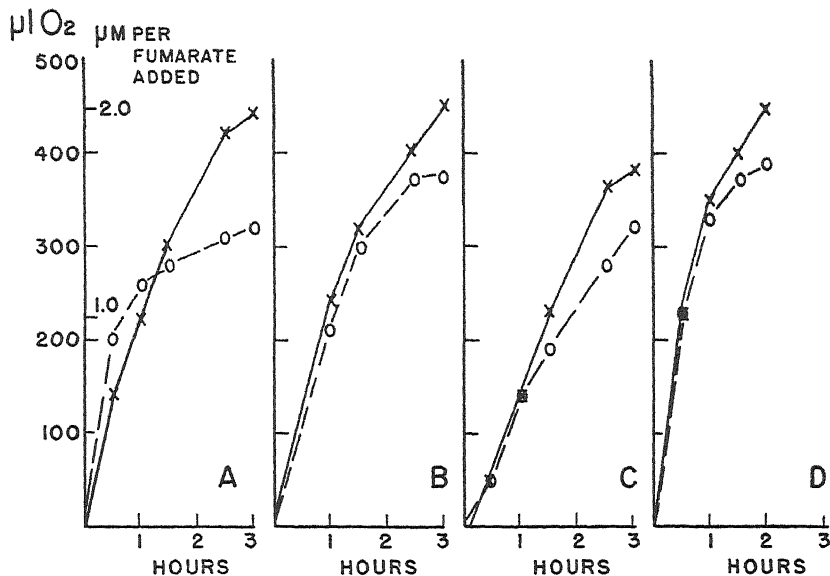


FIG. 4. Oxidation of fumarate by cells grown under different conditions. All oxidations in 0.003 M phosphate buffer, pH 7, 1 mg. of bacterial nitrogen per cup; streptomycin concentration when present, 10  $\gamma$  per ml. (broken lines). A, cells grown on Medium A, 30°, 16 hours; B, cells on Medium A, 37°, 16 hours; C, cells on Medium A plus 0.1 per cent glucose, 37°, 16 hours; D, cells on Medium B, 30°, 16 hours.

the respiration of fumarate itself may be observed. A comparison of the oxidation of fumarate by cells grown under various conditions is given in Fig. 4. While it is apparent that there is some variation in the rate and extent of fumarate oxidation, depending upon the medium, growth temperature, and doubtless other factors, there is also consistently less complete oxidation of fumarate in the streptomycin-treated cells. It is also apparent from Fig. 4 that the oxygen uptake in all conditions proceeds to at least 1 mole of  $\text{O}_2$  per mole of fumarate before a marked streptomycin effect is evi-

dent. The utilization of 0.5 mole of  $O_2$  per mole of fumarate would bring it to the oxidation state of pyruvate; hence there must be the utilization of 0.5 mole of  $O_2$  per mole of pyruvate (at least) before the streptomycin effect is noted. Fig. 5 shows that close to 0.5 mole of  $O_2$  per mole of pyruvate added is consumed before any great effect of streptomycin is observable.

The general conclusion that one may draw from these data is that streptomycin prevents the "terminal respiration" process in the susceptible

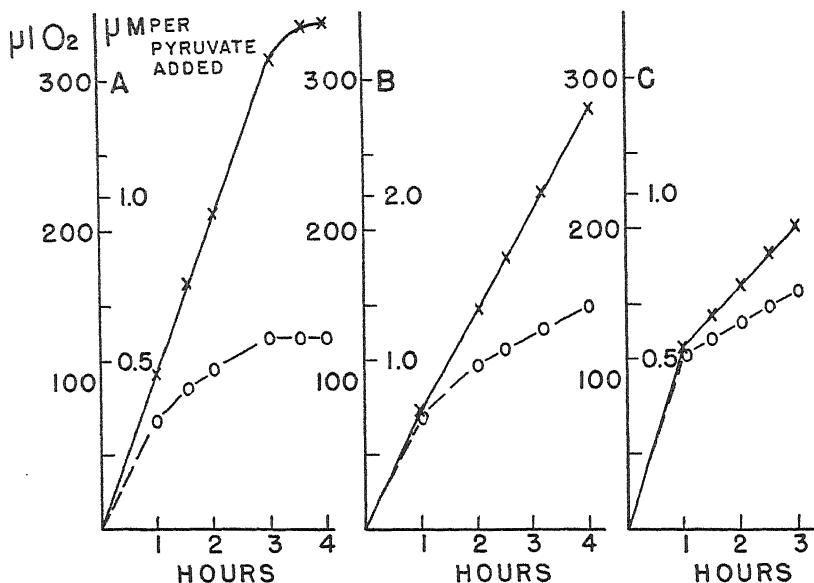


FIG. 5. Oxidation of pyruvate. All oxidations in 0.003 M phosphate buffer, pH 7. Streptomycin concentration when present, 10  $\gamma$  per ml. (broken lines). A, 1 mg. of bacterial nitrogen per cup, cells grown on Medium B, 30°, 18 hours, 10 micromoles of pyruvate added; B, 0.45 mg. of bacterial nitrogen per cup, cells on Medium B, 30°, 16 hours, 5 micromoles of pyruvate added; C, 1.0 mg. of bacterial nitrogen per cup, cells on Medium A, 30°, 18 hours, 10 micromoles of pyruvate added.

bacteria. This is true of threonine (or the keto acid derived from it), in which a previous oxidation of fumarate was necessary before it could enter the terminal respiration system, as well as for fumarate and pyruvate in which only a partial oxidation is observed when streptomycin is present. For investigation of the terminal respiration process in this strain, two possibilities were considered.

First, the data on pyruvate oxidation shown in Fig. 5 suggest that pyruvate is oxidized to the acetate stage. Thereafter, when streptomycin is present, further oxidation is inhibited. Acetate itself is not readily oxi-

dized by this strain and what oxidation does occur is only slightly affected by streptomycin. In fact, the rate of oxidation observed with pyruvate and streptomycin (Fig. 5) after the 0.5 mole of  $O_2$  per mole of pyruvate point corresponds roughly with the ability of the suspensions to oxidize added acetate. Nevertheless, one could postulate that an "active acetate" was the result of pyruvate oxidation, and that it was this material which was further oxidized. According to this hypothesis, streptomycin would prevent the formation of "active acetate."

Second, the data of Fig. 5 could equally well suggest that, in the absence of streptomycin, the pyruvate was oxidized by a process similar to terminal respiration in the animal; *i.e.*, condensation with oxalacetate to enter the tricarboxy acid cycle (12). If this process were inhibited by streptomycin, the pyruvate could be oxidized by a different pathway to the acetate stage. The essential difference between these hypotheses is that the first considers that the initial stages of the process of pyruvate oxidation are the same in the presence or absence of streptomycin, whereas the second considers that two different processes are involved.

Search for the formation of "active acetate" was not experimentally successful. In our hands, the hydroxylamine method of Lipmann and Tuttle (9) has not revealed "active acetate" (not necessarily acetyl phosphate) at any stage of the process. Recourse was therefore had to vacuum-dried cell preparations in the hope that conditions could be obtained in which some of the side reactions were eliminated. In the case of the reactions studied here, the dried preparations were relatively unstable and retained the activities described for about 24 hours, thereafter becoming completely inactive. As might be expected, occasional preparations were entirely inactive. Data from active preparations were typical of those shown in Fig. 6. In this case, as for other preparations, pyruvate could be replaced by threonine with essentially the same results except that the reactions were somewhat slower.

The first portion of Fig. 6 shows that when fumarate is added there is a lag period of approximately 90 minutes before the maximum rate of oxidation is observed. During this period there is a gradually increasing rate of oxygen uptake and by the time the maximum rate has been reached (90 minutes) 0.5 mole of  $O_2$  per mole of fumarate has been used. This would be sufficient to oxidize the fumarate to the oxalacetate stage. One may presume that the rate of oxidation thereafter is controlled by the oxalacetate and pyruvate (its decarboxylation product) condensations and indeed, since the addition of pyruvate at 3 hours has no effect, the rate-controlling factor is probably oxalacetate. When streptomycin is present, there is a slight initial oxidation which soon stops, and addition of pyruvate at 3 hours has no effect.

In the second portion of Fig. 6, the addition of pyruvate causes first an oxygen uptake essentially uninfluenced by streptomycin except in the later stages. However, the oxygen taken up falls far short of that necessary for oxidation even to the acetate stage. The addition of fumarate now results in a rapid oxidation with a lag period of only 30 minutes and the uptake of only 20 microliters of  $O_2$  before the maximum rate is reached. When streptomycin is present, fumarate does not initiate any oxidation.

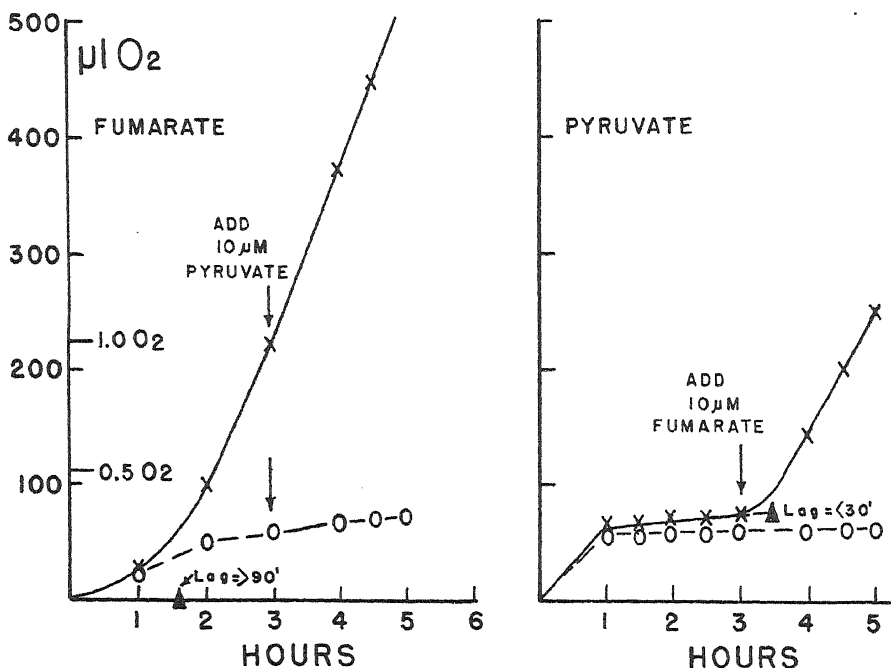


FIG. 6. Oxidation of fumarate and pyruvate by dried preparations of *Escherichia coli*. Each flask contained 20 mg. of vacuum-dried preparation of cells of *E. coli* grown on Medium A, 30°, for 16 hours, 0.003 M phosphate buffer, pH 7; 10 micromoles of fumarate or pyruvate added as indicated. Streptomycin when present at 20 γ per ml. (broken lines).

These results point definitely to the second hypothesis; that is, that pyruvate can be oxidized by way of condensation with oxalacetate, and that in dried cell preparations, in which the oxidation to acetate is weak, the condensation reaction is the principal pathway. Streptomycin prevents the condensation reaction. This mode of action of streptomycin appears to be relatively clear. However, the mechanism by which it prevents this condensation is still obscure. The data of Fig. 3 demonstrate that

streptomycin prevents the *formation* and not the activity of the substance which is formed from fumarate oxidation. The data of Fig. 6 indicate this substance to be oxalacetate. In addition the data of Fig. 1 show that at higher phosphate levels the oxidation of fumarate proceeds at the same rate with or without streptomycin; yet the ability of the cell to oxidize threonine is impaired when streptomycin is present. Until these reaction systems are untangled experimentally, it is not safe to ascribe the inhibition exerted by streptomycin to any stage in this evidently complex process. Thus from these experiments it is permissible to conclude only that the net result of streptomycin inhibition is to prevent pyruvate (and evidently other keto acids) from entering the terminal respiration system by way of oxalacetate condensation. It is further evident that, since the observable reaction inhibited is also an important one in animal metabolism, it is as yet difficult to explain why the streptomycin is able to kill the bacteria without comparable harm to the host. At least two possibilities, that streptomycin does not penetrate to the active centers of this reaction in the host, or that the condensation in the bacterium differs in some subtle manner from that occurring in the host, are matters for further study.

#### SUMMARY

1. In the Gratia strain of *Escherichia coli* the oxidation of threonine is stimulated by the previous oxidation of fumarate.
2. This stimulating effect is prevented by streptomycin.
3. Streptomycin can be shown to inhibit the oxidation of fumarate and pyruvate under suitable conditions.
4. The mode of action of streptomycin is to inhibit the terminal respiration process.
5. The terminal respiration process apparently involves a pyruvate-oxalacetate condensation and it is close to this reaction that streptomycin exerts its activity.

Thanks are due Mr. James G. Waddell for technical assistance.

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# SOLUBILITY OF GLYCINE, LEUCINE, AND TYROSINE IN LIQUID AMMONIA

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(Received for publication, October 4, 1948)

The literature apparently contains no quantitative data on the solubility of amino acids in liquid ammonia. Franklin and Kraus (1) list glycine, leucine, tyrosine, and alanine as "very soluble" in commercial liquid ammonia. In this investigation the solubilities of the first three of these compounds in anhydrous ammonia were determined at four temperatures between  $-35^{\circ}$  and  $-77^{\circ}$ .

## EXPERIMENTAL

Tank liquid ammonia (Ohio Chemical and Manufacturing Company) was distilled from sodium shavings into a tube of about 120 cc. volume. This was equipped with a thermocouple well, inlet and outlet side arms protected with calcium chloride and phosphorus pentoxide drying towers, and a standard taper cap containing a mercury seal stirrer. A current of dry nitrogen could be passed through the tube, over the liquid ammonia.

The amino acids were C.P. products (Interchemical Corporation). After drying 3 days in a vacuum desiccator, a sample of 10 to 15 gm. was added to the liquid ammonia. The solubility tube was surrounded by partially melted crystals of ethylene dichloride (m.p.  $-35.3^{\circ}$ ), chlorobenzene ( $-45.7^{\circ}$ ), chloroform ( $-63.5^{\circ}$ ), or solid carbon dioxide and acetone ( $-77^{\circ}$ ), depending on the constant temperature desired.

After stirring for 8 to 16 hours, the suspended particles were allowed to settle until the supernatant solution appeared perfectly clear. Then, while dry nitrogen was flowing, a glass dipper of calibrated volume was filled with the solution and placed in a test-tube immersed in frozen ethylene dichloride. Evaporation of ammonia proceeded slowly and without spattering of the solution. Finally, the solid residue was dried in the vacuum desiccator and weighed. Determinations were continued at intervals of several hours until there was no further increase in the amount dissolved.

Glycine, DL-leucine, and L-tyrosine dissolved to yield clear solutions. L-Glutamic acid and L-cystine formed oily liquids which on stirring went into suspension in the liquid ammonia phase. Most of this settled out on

\* From a dissertation presented by the Reverend John B. Zachary, in partial fulfilment of the requirements for the degree of Master of Science.

standing 12 hours, but it was impossible to obtain a clear supernatant solution.

Glutamic acid appeared to be converted into an ammonium salt, for the dried residue evolved ammonia on treatment with sodium hydroxide. In all the other cases, the amino acid was recovered unchanged from the ammonia solution.

### Results

The solubilities found for glycine, tyrosine, and leucine are summarized in Table I. The solubilities in water at 25° are given for comparison (2).

TABLE I  
*Solubilities in Liquid Ammonia (Moles per Liter of Solution)*

	Temperature, °K.				
	196	210	227.5	238	Water at 298.2
Glycine.....	0.20	0.65	2.52	3.95	2.90
DL-Leucine.....	0.050	0.053	0.055	0.057	0.08
L-Tyrosine.....	0.0021	0.0031	0.0118	0.0220	0.0025

TABLE II  
*Calculated Heat of Solution*

	$-\Delta H$ (solution in $\text{NH}_3$ )	$-\Delta H$ (solution in $\text{H}_2\text{O}$ at 25°)
	calories	calories
Glycine.....	7150	3750
DL-Leucine.....	340	2000
L-Tyrosine.....	7130	5950

If the logarithms of the solubilities are plotted against the reciprocal absolute temperature, moderately straight lines are obtained. From the slopes of the curves, the approximate values of the heat of solution shown in Table II can be calculated. The water values are also cited (2).

### SUMMARY

The solubilities of glycine, DL-leucine, and tyrosine in anhydrous liquid ammonia have been measured between  $-35^\circ$  and  $-77^\circ$ . The values are comparable with the solubilities in water at 25°.

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# INCORPORATION OF ISOTOPIC CARBON DIOXIDE IN RABBIT LIVER GLYCOGEN IN VITRO\*

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(Received for publication, October 7, 1948)

Previous preliminary studies have shown that isotopic carbon dioxide,  $C^{14}O_2$ , when incubated with rabbit liver slices in the presence of pyruvate as substrate and K ions, resulted in the synthesis of glycogen in which 7 to 16 per cent of the carbon atoms had the same specific radioactivity as the inorganic carbon of the medium (1).

In a critical study of the effects of cations on glycogen synthesis by liver slices *in vitro*, Buchanan found that an equimolecular mixture of sodium and potassium, supplemented by a divalent ion such as calcium or magnesium, was superior for glycogen synthesis to solutions containing either no sodium or no potassium (2). The present work was undertaken to determine by the use of  $C^{14}O_2$  (1) the extent to which  $CO_2$  participated in glycogen synthesis *in vitro*, and (2) whether variations in the cationic composition of the medium influenced the incorporation of  $CO_2$  in the course of glycogen synthesis. Evidence has also been obtained on livers of fed rabbits that glycogen synthesis is proceeding *in vitro* even in the face of a net decrease in the glycogen content of the liver.

## EXPERIMENTAL

The experimental procedures employed were similar to those previously reported (1). The three incubation media used were prepared and had the ionic composition shown in Table I. The solutions were then equilibrated with 95 per cent  $O_2$ -5 per cent  $CO_2$ , which in the presence of the liver slices resulted in pH values between 7.4 and 7.5.

Rabbits, fasted for 48 hours, were killed, and the livers were removed and sliced with a Stadie slicer as rapidly as possible. The slices, 0.5 mm. thick, were put into an appropriate slicing medium which was identical with the incubation medium, except for the omission of Ca and pyruvate.

2 to 5 gm. of liver slices were then transferred to a 150 cc. Soxhlet flask containing 25 cc. of incubation medium and closed with a 2-hole rubber stopper fitted with two capillary stop-cocks (2 mm. inside diameter). One

\* This work was supported in part by a contract between Harvard University and the Office of Naval Research, and in part by a grant-in-aid from Swift and Company, Inc.

Unless otherwise specified, the flasks were then gently shaken for 2 hours, during which samples of the liquid phase were removed from time to time for CO<sub>2</sub> analyses in the Van Slyke manometric apparatus. This CO<sub>2</sub> was then liberated in the Van Slyke chamber and transferred to 1 cc. of satu-

Stock solution	Na medium	K medium	Na-K medium
1 N HCl, ml.....	6.5	6.5	6.5
1 M NaHCO <sub>3</sub> , ml.....	10.5	0	3.2
1 " KHCO <sub>3</sub> , " .....	0	10.5	7.3
0.1 M CaCl <sub>2</sub> , ml.....	10	10	10
Na pyruvate·H <sub>2</sub> O, mg.....	512	0	512
K " " .....	0	576	0
Phenol red, 0.1%, ml.....	0.76	0.76	0.76
H <sub>2</sub> O to make, ml.....	100	100	100
Cations*			
Na.....	145	0	72
K.....	0	145	73
Ca.....	20	20	20
Anions*			
Cl.....	85	85	85
HCO <sub>3</sub> .....	40	40	40
Pyruvate.....	40	40	40

At the end of the experiment, 80 per cent KOH solution was added and the tissue digested. From the digest, aliquots were taken for phosphate and glycogen analyses and from the remainder glycogen was isolated. Total phosphorus was determined by the Fiske and Subbarow method (3), and the wet weight of tissue was calculated by multiplying the mg. of

phosphorus by 250. Glycogen was determined by the method of Good, Kramer, and Somogyi (4) as modified by Sjögren (5). The isolated glycogen was purified for radioassay by extraction with ice-cold 5 per cent trichloroacetic acid and by precipitation with 60 per cent alcohol. This served to remove any radioactive carbonates still present in the glycogen and gave a pure white preparation.

*Radioactive Assay*—The measurement of the radioactivity of the  $C^{14}$  in the  $BaCO_3$  and glycogen preparations was carried out as described by Solomon *et al.* (6). A Geiger-Müller counter with a mica end window of 1.5 to 2.5 mg. per sq. cm. thickness was used. The  $BaCO_3$  or glycogen sample (about 5 mg. in size) was suspended in a few drops of water and transferred to a stainless steel cup containing a well 1 mm. deep  $\times$  14 mm. in diameter cut in the top surface; the suspension was stirred until homogeneous and allowed to evaporate to dryness slowly, giving a uniform layer with a density of about 3.24 mg. per sq. cm. The amount of  $BaCO_3$  was calculated from the total  $CO_2$  as measured gasometrically, and in most cases it was also checked gravimetrically.

A series of ten samples of isotopic  $BaCO_3$ , prepared as described and analyzed for  $CO_2$  and  $C^{14}O_2$ , gave specific activities for  $C^{14}$  with a reproducibility within 1.5 per cent, including counting errors.

*Background*—Background counts were measured for 30 to 60 minutes, at least twice and usually three times a day. Due to shielding by 2 inch thick lead bricks and the characteristics of the counter used, the background was low and constant, usually remaining within  $\pm 1$  count per minute for any one counter during 1 day.

*Self-Absorption*—The self-absorption curve for  $BaCO_3$  was determined over the range 0.6 to 13 mg. per sq. cm., and the amount of self-absorption at a density of 3.24 mg. per sq. cm. (*i.e.*, 5 mg. per cup) was taken arbitrarily as a standard. Counts for samples differing from this density were corrected by multiplying by a factor obtained from the self-absorption curves.

The glycogen samples were prepared by evaporating a solution in distilled water to dryness in the cup. The amount of glycogen was determined by analysis of the solution and also checked by weight. As would be anticipated, self-absorption studies on glycogen over the range 0.9 to 9 mg. per sq. cm. gave a self-absorption curve indistinguishable from that given by  $BaCO_3$  over a comparable range. The validity of this procedure was confirmed experimentally by comparison of the specific activity of glycogen measured as such and as  $BaCO_3$  (subsequent to combustion). Agreement within 5 per cent was obtained.

In the present experiments, only the ratios of the counts per minute of

the  $BaCO_3$  derived from the liquid phase to the counts per minute of the glycogen samples are used in calculating the per cent incorporation of  $CO_2$ . Hence, it was unnecessary to correct for the geometry of the system.

*Calculation of Per Cent Incorporation of  $CO_2$* —The corrected counts per minute divided by the mm of inorganic carbon in the sample counted gave the specific activity of the  $CO_2$  carbon in the liquid phase. The specific activity of the inorganic carbon at the mid-point of the incubation period (usually 1 hour) was taken as representing the average specific radioactivity of inorganic carbon of the milieu in which the glycogen was formed.

The corrected counts per minute divided by the mm of glycogen carbon in the sample counted gave the specific activity of the carbon atoms in the glycogen. The specific activity of the glycogen carbon divided by the mean specific activity of the inorganic carbon, times 100, gives the per cent of glycogen carbon atoms which had once been inorganic carbon in the system.

### Results

The per cent incorporation of inorganic carbon into liver glycogen was first studied in a medium containing equimolecular amounts of Na and K ions. In a series of seven experiments on fasted rabbits, the incorporation was between 5.2 and 9.9 per cent, averaging 7.9 percent (Table II).

The per cent incorporation of inorganic carbon into glycogen ( $(b/a) \times 100$ ) was calculated on the basis that all the glycogen found at the end of the incubation period was newly formed glycogen. The small amount of glycogen initially present undoubtedly had been turned over to a considerable extent (7), so that the figure of 7.9 per cent is taken to be the average per cent of incorporation of inorganic carbon in all new glycogen formed in this medium.

In a second series of experiments differing only in that the livers contained high initial levels of glycogen, there was usually a net decrease in glycogen content during the incubation period, but some incorporation of  $C^{14}O_2$  into glycogen nevertheless was found (Table III), indicating that glycogenesis was proceeding, though at a slower rate than glycogenolysis. In the two experiments (Nos. 1 and 2) in which slices from the same liver were incubated for varying lengths of time, the per cent incorporation is seen to increase with time.

*Influence of Cations on  $CO_2$  Incorporation*—Since it has been previously observed in this laboratory (8) that an incubation medium rich in potassium favored glycogenesis whereas a medium rich in sodium and low in potassium (as in Ringer's solution) favored glycogenolysis, the effect of varying concentrations of these 2 cations on  $CO_2$  incorporation in glycogen was studied. A comparison was made of the per cent  $CO_2$  incorporation when

the liver slices were incubated in the Na medium, K medium, and Na + K medium. Table IV contains the results of six such experiments.

It was observed that when the initial glycogen content of the liver was low (Experiments 1 to 3) the chemically determined final glycogen ex-

TABLE II

*Incorporation of  $C^{14}O_2$  in Liver Glycogen of Fasted Rabbits with Na-K Pyruvate Medium*

Incubation time, 2 hours.

Experiment No.	Glycogen, per cent wet weight		Specific activity, counts per min. per mm C $\times 10^{-3}$		Per cent incorporation $\frac{(b)}{(a)} \times 100$
	Initial	Increase	Inorganic carbon (a)	Glycogen carbon (b)	
1	0.08	0.40	15.1	1.50	9.9
2	0.04	0.46	16.0	1.41	8.8
3	0.02	0.19	35.0	3.42	9.8
4	0.02	0.35	31.0	1.97	6.4
5	0.03	0.25	28.6	1.49	5.2
6	0.02	0.50	69.2	5.94	8.6
7	0.10	0.36	37.5	2.46	6.6
Average....					7.9

TABLE III

*Incorporation of  $C^{14}O_2$  in Glycogen-Rich Rabbit Liver Slices, in Vitro, with Na-K Pyruvate Medium*

Experiment No.	Incubation time	Glycogen, per cent wet weight		Specific activity, counts per min. per mm C $\times 10^{-3}$		Per cent incorporation $\frac{(b)}{(a)} \times 100$
		Initial	Net change	Inorganic carbon (a)	Glycogen carbon (b)	
	<i>hrs.</i>					
1a	2	2.20	-0.28	101	1.39	1.4
1b	4	2.20	+0.07	79.5	1.83	2.3
2a	2	6.50	-0.4	170.3	0.67	0.4
2b	5	6.50	-1.0	141.1	1.12	0.8
3	2	5.40	-0.18	74.0	1.94	2.6
4	2	1.26	-0.34	75.0	2.74	3.65
5	2	1.85	-0.51	57.9	0.32	0.6
6	2	3.05	-0.69	21.5	0.32	1.5

ceeded the initial glycogen after incubation in both the K medium and the Na + K medium and was essentially unchanged in the Na medium experiments; when the initial glycogen was high (Experiments 4 to 6), the final glycogen was less than the initial glycogen in all solutions. However, the

net loss of glycogen was consistently greater in the Na medium than in the two media containing K. This confirms the effect previously observed of K ions in favoring glycogenesis (8).

The per cent incorporation of CO<sub>2</sub> was larger in the Na + K medium than in the K medium, indicating that more rapid turnover of glycogen is favored by the presence of the 2 cations. (In Experiments 1 to 3, the estimation of CO<sub>2</sub> incorporation in the Na medium experiments is subject

TABLE IV

*Effect of Na and K Ions in Incubation Medium on CO<sub>2</sub> Incorporation into Glycogen Substrate, pyruvate; Incubation time, 2 hours.*

Experiment No.	Solution	Glycogen, per cent wet weight		Specific activity, counts per min. per mm C $\times 10^{-3}$		Per cent incorporation $\frac{(b)}{(a)} \times 100$
		Initial	Net change	Inorganic carbon (a)	Glycogen carbon (b)	
1	Na	0.03	+0.02	38.0	0.36	0.9
	K	0.03	+0.07	34.0	0.84	2.5
	Na + K	0.03	+0.25	28.6	1.49	5.2
2	"	0.10	-0.02	35.3	2.16	6.1
	K	0.10	+0.17	40.5	1.62	4.0
	Na + K	0.10	+0.36	37.5	2.46	6.6
3	"	0.27	-0.04	117	7.8	6.7
	K	0.27	+0.36	137	5.8	4.2
	Na + K	0.27	+0.34	123	5.7	4.6
4	"	1.26	-0.51	85	2.2	2.6
	K	1.26	-0.29	77	2.0	2.6
	Na + K	1.26	-0.34	75	2.7	3.6
5	"	3.05	-2.67	18.7	0.07	0.4
	K	3.05	-0.52	36.2	0.27	0.7
	Na + K	3.05	-0.69	21.5	0.32	1.5
6	"	3.22	-1.92	55.5	0.03	0.1
	K	3.22	-0.08	49.5	0.34	0.7
	Na + K	Lost				

to considerable doubt due to the small quantities of glycogen available for chemical and radioassay. No conclusions are drawn at this time regarding the effect of Na ions alone on CO<sub>2</sub> incorporation.)

These observations point to the existence of an important relation between the enzymatic ionic environment and the activity of the enzymes participating in glycogen synthesis and breakdown. Since potassium is normally present in the intracellular fluid of liver in a concentration of about 140 milliequivalents per kilo of intracellular water, and since this intracellular potassium tends to exchange with extracellular ions upon incubation of liver *in vitro*, our results are interpreted as indicating that the

maintenance of a high intracellular potassium concentration is essential for glycogen synthesis and storage. It is of significance, in this connection, that Lardy and Ziegler have demonstrated the importance of potassium ions in favoring phosphorylation of pyruvate. A study of the effect of high potassium ion concentrations on other specific reactions concerned with glycogen synthesis would seem to be desirable.

*Glucose As Substrate*—Two experiments on the incorporation of  $C^{14}O_2$  in glycogen formed from glucose *in vitro* were carried out. Previous results

TABLE V  
*Comparison of Pyruvate and Glucose As Substrates on Incorporation of  $C^{14}O_2$  in Glycogen*

Experiment No.	Substrate	Glycogen, per cent wet weight		Specific activity, counts per min. per mm C $\times 10^{-3}$		Per cent incorporation $\frac{(b)}{(a)} \times 100$
		Initial	Net change	Inorganic carbon (a)	Glycogen carbon (b)	
1a	Pyruvate	0.02	+0.35	31.0	1.97	6.4
1b	Glucose	0.02	+0.60	41.4	0.78	1.9
2a	Pyruvate	3.22	-1.09	68.1	0.30	0.44
2b	Glucose	3.22	-0.01	70.8	0.18	0.26

TABLE VI  
*Incorporation of  $C^{14}O_2$  in Glycogen in Rat Liver Slices*  
Substrate, pyruvate; incubation time, 2 hours.

Experiment No.	Glycogen, per cent wet weight		Specific activity, counts per min. per mm C $\times 10^{-3}$		Per cent incorporation $\frac{(b)}{(a)} \times 100$
	Initial	Net change	Inorganic carbon (a)	Glycogen carbon (b)	
1	0.15	-0.04	66.3	5.69	8.6
2	0.96	-0.66	62.6	1.12	1.8

from this laboratory had shown that some but considerably less incorporation of  $CO_2$  occurs with glucose as substrate than with pyruvate (9). This finding was confirmed, as is shown in Table V, indicating that, even with glucose as added substrate, the turnover of glycogen is sufficient to permit a considerable amount of pyruvate to be formed, made radioactive, and re-synthesized to glycogen.

*Glycogen Synthesis in Rat Liver Slices*—Although glycogen synthesis by rat liver slices from glucose *in vitro* has been demonstrated (8), attempts to obtain evidence for glycogen synthesis from pyruvate by rat liver have hitherto been unsuccessful. Six rats were studied by the method described

above, and from two of them small but sufficient amounts of glycogen were obtained after incubation to permit isolation in pure form. The per cent incorporation of  $CO_2$  observed (Table VI) is similar to that found for rabbit liver, even though there was a net decrease in liver glycogen in each instance.

#### DISCUSSION

It was suggested in a previous report from this laboratory (10) that a reasonable explanation for the experimental results on incorporation of  $CO_2$  into liver glycogen could be found in the formation of phosphopyruvate from pyruvate via an indirect pathway involving  $CO_2$  fixation and the phosphodicarboxylic acids, phosphomalate and phosphooxalacetate. Since the demonstration by Lardy and Ziegler (11) that the reaction, phosphopyruvate + adenosine diphosphate  $\rightleftharpoons$  pyruvate + adenosine triphosphate, is reversible (in the presence of K ions), another explanation for the appearance of labeled  $CO_2$  in glycogen would equally well account for the experimental results and on the basis of existing evidence is the more probable.

This is simply that carboxyl-labeled pyruvate molecules are formed by (a) carboxylation of pyruvate with isotopic  $CO_2$  to form  $\beta$ -carboxyl-labeled oxalacetate, (b) enzymatic equilibrium of oxalacetate with the symmetrical dicarboxylic acids which would distribute the isotopic C between both oxalacetate carboxyl groups, and (c) decarboxylation of oxalacetate which would yield carboxyl-labeled pyruvate from half of the labeled oxalacetate molecules (12, 13).

Subsequent repetitions of these reactions might be expected to yield pyruvate, all of whose carboxyl groups had the same specific activities as the inorganic carbon. If such pyruvate molecules were the sole source of glycogen, one might expect 33 per cent incorporation of  $CO_2$  in the glycogen, as pointed out by Wood, Lifson, and Lorber (14).

Since the observed amount incorporated is only 8 per cent in the present experiments *in vitro* and averaged only 11 or 12 per cent in the previous experiments *in vitro* and *in vivo* (1, 9, 10), either the pyruvate carboxyls may not have been in equilibrium with the inorganic carbon or a fraction of the glycogen carbons may have been derived from carbon precursors other than the pyruvate carbons. Evidence will be presented in the following paper that the equilibration of the pyruvate carboxyls with  $CO_2$  is rapid compared with the conversion of pyruvate to glycogen.

The rôle of the intracellular ionic environment in influencing the balance between glycogenic and glycogenolytic reactions has again been demonstrated: potassium ions favor the former and sodium ions the latter. It is, therefore, not without physiological interest that the rates of incorpora-



tion of CO<sub>2</sub> were, in general, greatest in those experiments in which the incubating medium contained both ions in equal proportions. The factors which control or modify the composition of the intracellular ionic environment and thereby affect the activity of intracellular enzymes merit further investigation.

The amount of new glycogen present at the end of the incubation period has been estimated in those experiments in which there was a net decrease in chemically determinable glycogen. This was done for Experiments 1, 2, 4, and 6 of Table III, assuming that 8 per cent CO<sub>2</sub> incorporation would represent 100 per cent new glycogen, according to the following equation:

$$\text{Weight of new glycogen} = \text{weight of final glycogen} \times \frac{\% \text{ CO}_2 \text{ incorporation found}}{8}$$

(gm. per 100 gm. liver)                      (gm. per 100 gm. liver)

The average weight of new glycogen formed per 100 gm. of liver amounted in these experiments to 0.37 gm. in 2 hours. This value is essentially the same as the average net increase in liver glycogen formed in the experiments on fasted animals cited in Table II. When similar estimates are made on the effect of Na and K on the amount of new glycogen formed, the value for the Na medium is 0.1 gm., for the K medium, 0.21 gm., and for the Na-K medium, 0.37 gm. It would, therefore, appear that glycogen was synthesized at approximately the same rates under our experimental conditions (in the Na-K medium), regardless of whether the livers were originally rich or poor in glycogen.

#### SUMMARY

1. Glycogen formation and C<sup>14</sup>O<sub>2</sub> incorporation have been studied in rabbit liver slices *in vitro*, with pyruvate as substrate and with variations in sodium and potassium concentrations in the incubating media.

2. The incorporation of CO<sub>2</sub> averaged about 8 per cent in the liver glycogen of fasted animals. It was less in well fed animals, or when glucose was substituted for pyruvate as substrate.

3. Potassium ions favored glycogenesis and sodium ions glycogenolysis. The amount of glycogen formed during incubation and the extent of CO<sub>2</sub> incorporation in glycogen was usually greater when both ions were present in equimolecular proportions than when each was present alone.

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# METABOLISM OF ISOTOPIC PYRUVATE AND ACETATE IN RABBIT LIVER SLICES IN VITRO\*

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(Received for publication, October 7, 1948)

In the preceding paper, it was reported that incubation of liver slices from fasted rabbits with pyruvate in the presence of  $C^{14}O_2$  resulted in the formation of liver glycogen in which the specific activity of the carbon atoms was, on the average, 8 per cent of the specific radioactivity of the inorganic carbon of the medium (1). In the present paper, comparisons are made of the incorporation of  $C^{14}$  in glycogen from isotopic bicarbonate, isotopic pyruvate ( $\alpha$ -carbon-labeled), and isotopic acetate (carboxyl carbon-labeled). Liver slices from the same animal were used for the comparison and the experiments were carried out at the same time and in the same manner, except for the difference in labeled substrate. Unlabeled pyruvate was present in all experiments.

The purpose of these experiments was two-fold: (1) to compare the radioactivity of the glycogen derived from  $\alpha$ -labeled pyruvate with that derived from unlabeled pyruvate and labeled  $CO_2$ , and (2) to determine the extent to which acetate carbons were incorporated into glycogen without previously being metabolized to  $CO_2$ .

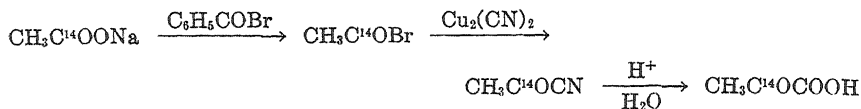
From the experiments with labeled pyruvate and labeled  $CO_2$ , it is concluded that in livers rich in glycogen the pyruvate molecules are brought into equilibrium with the dicarboxylic acids much more rapidly than they are synthesized to glycogen.

## EXPERIMENTAL

*Synthesis of Isotopic Acetate and Pyruvate*—(a) Carboxyl-labeled acetate was prepared by the method of Sakami, Evans, and Gurin (2). The product assayed  $28.6 \times 10^6$  counts per minute per mm of carboxyl carbon.

\* This work was supported in part by a contract between Harvard University and the Office of Naval Research, and in part by a grant-in-aid from Swift and Company, Inc.

(b) Pyruvic acid labeled in the  $\alpha$ -carbon atom was synthesized by the following sequence of reactions:



A similar synthesis was recently briefly reported by Calvin and Lemmon (3).

*Acetyl Bromide*—10 mg. of fused isotopic sodium acetate, 1.5 gm. of fused non-isotopic sodium acetate, and 7 ml. of benzoyl bromide were added to a small distilling flask and heated at 175° in an oil bath. The crude acetyl bromide (1.6 gm.) which distilled over was not purified but was used directly in the succeeding reaction.

*Pyruvitrile*—Acetyl bromide (1.6 gm.) was converted into pyruvitrile by treatment with cuprous cyanide according to the method described by Tschelinzeff and Schmidt (4). The crude product (0.85 gm.) was hydrolyzed without further purification.

*Pyruvic Acid*—The hydrolysis of pyruvitrile (0.85 gm.) was also conducted according to the method of Tschelinzeff and Schmidt (4). Before the ether was evaporated, 0.3 ml. of non-isotopic pyruvic acid was added as a carrier. The residue obtained after removal of the ether was fractionated in a small Claisen flask as follows: The flask was immersed in a water bath at 55° for 10 minutes while the pressure was maintained at 10 mm. After thus removing any acetic acid that might have been formed in the hydrolysis, pyruvic acid was collected when the bath temperature was raised to 90°. The purity of the product was determined by measuring its index of refraction, assuming any impurity present to be acetic acid. The material obtained in this way was 97 to 98 per cent pure. The free acid was converted to its sodium salt by treatment with aqueous sodium bicarbonate. The salt was crystallized from ethanol-acetone after the removal *in vacuo* of most of the water. Yield, 750 mg. of sodium pyruvate. The radioactivity of the  $\alpha$ -carbon atom was 19,170 counts per minute per mm of carbon.

*Media*—The Na-K media used for the  $\text{C}^{14}\text{O}_2$  and labeled acetate experiments were prepared the same way as previously described (1). The isotopic bicarbonate and acetate were added in a small volume of water subsequent to equilibration. The labeled acetate added amounted to only about 0.005 mm of highly active acetate. The medium for the labeled pyruvate experiments differed only in that the pyruvate was replaced by an equal amount of  $\alpha$ -labeled pyruvate. Liver slices from unfasted rabbits were pooled and 2 to 4 gm. amounts were put in each reaction flask.

In most of the experiments, duplicate flasks were incubated for 2 and 4 to 5 hour periods. The technique of equilibration and the analytical procedures were identical with those described in the preceding paper (1).

### Results and Comment

Table I gives the results of the experiments in which  $C^{14}O_2$  incorporation was compared with that of  $CH_3C^{14}O_2COOH$ . The per cent incorporation of  $C^{14}O_2$  was low (Column 10, *a*), due to the high initial glycogen levels and the consequent dilution of newly formed glycogen with existing glycogen.

TABLE I

*Comparison of  $C^{14}$  Incorporation in Rabbit Liver Glycogen from  $C^{14}O_2$  and  $CH_3C^{14}O_2COOH$*

Na-K medium; substrate, 40 mm of pyruvate per liter.

Ex- peri- ment No.	Isotopic substrate	Time of incu- bation	Glycogen, per cent wet weight		CO <sub>2</sub> specific activity at mid- point, counts per min. per mm C $\times 10^{-3}$	Glycogen specific activ- ity, counts per min. per mm C $\times 10^{-3}$		Pyruvate specific activity, counts per min. per mm pyruvate $\alpha$ -C corrected for dilution, $\times 10^{-3}$	Incorporation of (a) CO <sub>2</sub> -C or (b) pyruvate $\alpha$ -C	Ratio, $\alpha$ -C incorporation CO <sub>2</sub> -C incorporation
			Initial	Net change		Found	Cor- rected for CO <sub>2</sub> incor- pora- tion (8)			
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
		<i>min.</i>							<i>per cent</i>	
1a	CO <sub>2</sub>	120	6.5	-0.4	170	0.67			(a) 0.39	
1b	Pyruvate	120	6.5	-0.5	1.05	0.056	0.052	17.91	(b) 0.29	0.74
1c	CO <sub>2</sub>	300	6.5	-1.0	141	1.12			(a) 0.79	
1d	Pyruvate	300	6.5	-1.6	1.76	0.15	0.136	16.62	(b) 0.82	1.04
2a	CO <sub>2</sub>	138	5.4	-0.2	74	1.94			(a) 2.6	
2b	Pyruvate	130	5.4	-0.05	1.3	0.48	0.45	19.08	(b) 2.4	0.92
2c	"	132	5.4	-1	1.5	0.35	0.31	19.08	" 1.6	0.62
Average.....										0.83

The incorporation of pyruvate (Column 10, *b*) was calculated on the basis of the average specific activity of glycogen carbon atoms divided by the specific activity of the  $\alpha$ -carbon atoms of pyruvate. Correction has been made for the incorporation of  $C^{14}O_2$  formed from oxidation of pyruvate during the experiment. (The rate of oxidation of pyruvate was only about 4 mg. per hour per gm. of tissue (wet weight).) A correction was also made for the dilution of isotopic pyruvate with the carbohydrate added to the medium as the result of glycogenolysis during the incubation. This correction was found to be small even if the assumption is made that all

the glycogen which disappeared was converted into pyruvate, or passed through pyruvate.

In Column 11 of Table I, the ratio of pyruvate  $\alpha$ -carbon incorporation to  $\text{CO}_2$  incorporation has been calculated. These ratios varied from 0.6 to 1, averaging 0.83. It would appear from these results that approximately 1 mole of  $\text{CO}_2$  was incorporated into glycogen for each mole of pyruvate which was converted to glycogen. In other words, the enzymatic equilibration of the carboxyl groups of pyruvate with  $\text{CO}_2$  apparently occurred much more rapidly than did the reactions required for the conversion of pyruvate to glycogen.

TABLE II

Comparison of  $\text{C}^{14}$  Incorporation in Rabbit Liver Glycogen from  $\text{C}^{14}\text{O}_2$  and  $\text{CH}_3\text{C}^{14}\text{OOH}$  Na-K medium; substrate, 40 mm of pyruvate per liter.

Experiment No.	Isotopic substrate	Time of incubation	Glycogen, per cent wet weight		$\text{CO}_2$ specific activity at mid-point, counts per min. per mm C $\times 10^{-3}$	Glycogen specific activity, counts per min. per mm C $\times 10^{-3}$		Per cent incorporation of $\text{CO}_2$ in glycogen
			Initial	Net change		Found	Corrected for $\text{CO}_2$ incorporation	
		min.						
1a	$\text{CO}_2$	130	1.85	-0.55	57.9	0.32		0.55
1b	Acetate	130	2.05	-1.21	2.74	0.32	0.30	
2a	$\text{CO}_2$	124	2.20	-0.3	101	1.39		1.37
2b	Acetate	123	2.20	-0.1	3.76	0.22	0.17	
3a	$\text{CO}_2$	240	2.20	+0.07	79.5	1.83		2.3
3b	Acetate	240	2.20	+0.01	4.3	0.31	0.21	
4a	$\text{CO}_2$	130	5.41	-0.2	74.0	0.35		2.6
4b	Acetate	132	5.41	-1.6	3.84	0.35	0.25	
4c	"	236	5.41	-1.3	7.18	0.95	0.58	

In Table II is given the comparison of the results obtained with isotopic  $\text{CO}_2$  and carboxyl-labeled acetate in the presence of an excess of unlabeled pyruvate. These results show that, after allowing for the acetate metabolized to  $\text{CO}_2$  and correcting for  $\text{CO}_2$  incorporation, there was definite residual radioactivity in the liver glycogen. This is interpreted as confirming *in vitro* the observation made by Lorber, Lifson, and Wood (5), *in vivo*, that acetate may contribute its 2 carbon atoms, without combustion, to a glucose unit of glycogen. However, this does not imply a net increase in glycogen arising from acetate. Acetate may contribute carbon atoms to the glucose units of glycogen by pathways other than those of oxidation and  $\text{CO}_2$  fixation. Thus, acetate carbons may be precursors of glycogen carbons, but acetate is not necessarily a glycogen former, an important distinction which recently has been stressed by Bloch (6).

## SUMMARY

1. Carboxyl-labeled acetate and  $\alpha$ -carbon-labeled pyruvate have been prepared from  $C^{14}$ .

2. A comparison has been made of isotopic carbon incorporation in rabbit liver glycogen *in vitro* in the presence of (a)  $C^{14}O_2$ , (b) isotopic carboxyl acetate, and (c) isotopic  $\alpha$ -carbon pyruvate.

3. Evidence is presented that pyruvate molecules are equilibrated with  $CO_2$  far more rapidly than they are converted to glycogen in the liver of unfasted rabbits.

4. Evidence is also presented that acetate molecules may participate in the synthesis of glycogen *in vitro* without previous combustion to  $CO_2$ .

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# A STUDY OF TRANSMETHYLATION WITH METHIONINE CONTAINING DEUTERIUM AND C<sup>14</sup> IN THE METHYL GROUP\*

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(Received for publication, October 18, 1948)

The concept that the body is incapable of generating methyl groups for the methylation of certain nitrogen- and sulfur-containing compounds of the body but is dependent on the presence in the diet of methyl groups in a utilizable form such as in methionine and choline was first based on the classical type of nutritional experimentation (1). The basic experiment showed namely that choline plus homocystine appeared to be the dietary equivalent of methionine and that, without choline in the diet, homocystine could not support growth of the white rat. Indisputable proof of the transfer of methyl groups between choline and methionine was then established by the tracer technique. The methyl group was labeled with deuterium and its migration in the body from one compound to another was traced (2, 3). Various explorations of transmethylation which involved choline, betaine, methionine, creatine, anserine, dimethylaminoethanol, monomethylaminoethanol, sarcosine, and dimethylglycine were carried out employing this technique. The results were consistent with our hypothesis that the methyl group was transferred *in toto*. In fact, in one group of experiments the deuteriomethionine was fed for 94 days, and analysis of the choline and creatine of the tissues showed that approximately 88 per cent of the methyl groups of these compounds had been derived from the deuteriomethionine of the diet (3). It is obvious that, if the methyl group had been subjected to oxidation and reduction to effect the transfer with an intermediate such as formaldehyde, the value could not have exceeded 66 per cent, and if, in addition, the process were reversible, either directly or by some cycle, the value for the deuterium would have been even lower.

The possibility of labeling the methyl group with radiocarbon has now placed in our hands the opportunity of a direct test of these deductions. We have, therefore, carried out an experiment with methionine in which the methyl group has been labeled with radiocarbon and with deuterium,

\* The authors wish to express their appreciation to the Lederle Laboratories Division, American Cyanamid Company, for a research grant that has aided greatly in this work.

and the migration of the methyl group has been followed by the simultaneous measurement of the  $C^{14}$  and of the deuterium in the choline and creatine of the tissues after the feeding of the doubly labeled methionine. We have found that the ratio of deuterium to radiocarbon in the methyl groups of the choline and creatine is the same as in the methyl group of the dietary methionine within the experimental errors involved in the procedures.

This result offers a direct quantitative demonstration that, in the process of the transfer of the methyl group of methionine to choline and creatine, the hydrogen atoms of the methyl group do not exchange with the hydrogen atoms of the body water and that in this process the methyl group is transferred as a unit. This result also establishes a firm basis upon which to compare results obtained by the deuterium-labeling technique with those based on the labeling of the carbon itself, in so far as these compounds are concerned.

#### EXPERIMENTAL

*Deuterio-L-methionine* ( $CD_3SCH_2CH_2CHNH_2COOH$ )—This compound was prepared by the method of Patterson and du Vigneaud (4). S-Benzyl-L-homocysteine (5) was reduced with sodium in liquid ammonia. The resulting sodium derivative of L-homocysteine was methylated by the addition of deuteriomethyl iodide (2, 3). The deuterio-L-methionine was crystallized by dissolving it in a minimum amount of boiling water and adding 10 volumes of boiling ethanol. It was recrystallized once by the same procedure. The optical rotation was  $[\alpha]_D^{21} = -7.7^\circ$  ( $c = 1.0$ , in water).

$C_5H_{11}O_2NS$ . Calculated,<sup>1</sup> N 9.22, S 21.1; found, N 9.05, S 21.0

*Radio-L-methionine* ( $C^{14}H_3SCH_2CH_2CHNH_2COOH$ )—The radio-L-methionine used in this experiment was prepared by Melville, Rachele, and Keller (6). In that preparation the starting material was barium carbonate containing  $C^{14}$  which was purchased from the Clinton Laboratories of the Monsanto Chemical Company. Radiomethyl iodide was prepared from this barium carbonate by a series of reactions and was then used to methylate the sodium derivative of L-homocysteine in liquid ammonia.

*Feeding of L-Methionine Labeled in Methyl Group with Radiocarbon and Deuterium*—The doubly labeled methionine was prepared by mixing together 204.3 mg. (1.369 mm) of radio-L-methionine and 432.8 mg. (2.848 mm) of deuterio-L-methionine. On a molar basis the mixture contained 32.5 per cent radiomethionine and 67.5 per cent deuteriomethionine. In

<sup>1</sup> Calculated values are based on the increased molecular weight due to deuterium in the molecule.

order to obtain a homogeneous preparation the mixture was dissolved in the minimum amount of water, the water was then removed by evaporation, and the methionine was dried.

An adult male rat weighing 250 gm. was placed on a choline-free amino acid diet for a control period of 3 days. This diet had the following percentage composition: amino acid mixture 21.3 (1, 7), DL-methionine 1, L-cystine 0.5, sucrose 53.2, hydrogenated vegetable oil 19, corn oil 1, Osborne and Mendel salt mixture 4 (8). The diet also contained vitamins in the amounts included in a choline-free diet by Wilson and du Vigneaud (9).

At the end of the 3 day control period the rat was given the doubly labeled L-methionine in the diet at a level of 1 per cent, replacing the non-isotopic methionine. Except for this change the diet was the same as the control period diet. The isotopic methionine was fed for a period of 4 days. Since a previous experiment had demonstrated that the methyl group of methionine can be oxidized to carbon dioxide *in vivo* (10), the rat was kept for the 4 day period in an open circuit metabolism apparatus and the expired CO<sub>2</sub> was continuously collected in NaOH solution. During the 4 day period the rat consumed 59.3 gm. of the diet and gained 13 gm. At the end of this time the rat was killed.

*Isolation of Choline and Creatine*—Creatine was isolated from the carcass as creatinine potassium picrate (3). This derivative was recrystallized three times, and the purity was then demonstrated by the quantitative determination of the creatinine content with the Jaffe reaction.

Choline was isolated from the carcass as choline chloroplatinate (3). This was recrystallized three times from water and then analyzed.

$C_{10}H_{28}O_2N_2 \cdot PtCl_6$ . Calculated,<sup>1</sup> Pt 31.6; found, Pt 31.5

*Deuterium Analyses*—The deuteriomethionine and the choline and creatine derivatives were analyzed for deuterium by the falling drop method (11, 12). To insure the accuracy of these determinations a calibration curve was obtained at the same time with five freshly prepared, weighed dilutions of 99.9 per cent D<sub>2</sub>O with H<sub>2</sub>O. The dilutions ranged from 0.1 to 2.5 atom per cent D. For compounds with a higher deuterium content, a weighed sample of the compound was mixed with a weighed sample of pure palmitic acid before the combustion in order to bring the deuterium content of the water into this range. The analytical method was first checked by analysis of several compounds of known deuterium content. Because of the large amount of acid formed on burning the creatinine potassium picrate, the water obtained by combustion of this compound was repurified after the first density determination, and this

determination was then repeated. The average velocity of fall of the drop was unchanged on repurification, showing that the water was pure.

The values for the deuterium in the three compounds are given in column (A) of Table I. The deuterium content of the methyl groups of the compounds is recorded in column (B). These values were calculated on the assumption that all the deuterium was present in the methyl groups. That this assumption is justified in the case of choline was shown by an earlier experiment in which the isolated choline was degraded and all the deuterium was found to be present in the methyl groups (3). The deuterium content of the methyl group of the dietary doubly labeled methionine was calculated from the proportion of deuteriomethionine in it.

TABLE I

*Deuterium and C<sup>14</sup> in Dietary Methionine and Isolated Choline and Creatine*

Compound	Deuterium content		C <sup>14</sup> content		D in methyl group C <sup>14</sup> in methyl group group (B) (D)
	Compound (A)	Methyl group (B)	Compound (C)	Methyl group (D)	
	<i>atom per cent</i>	<i>atom per cent</i>	<i>counts per min. per mm</i>	<i>counts per min. per mm methyl</i>	
Radio-L-methionine			$1.124 \times 10^7$	$1.124 \times 10^7$	
Deuterio-L-methionine	24.85 $\pm 0.26$	91.10 $\pm 0.96$			
Doubly labeled L-methionine*		61.52 $\pm 0.65$		$3.650 \times 10^6$	$1.69 \times 10^{-5}$
Choline chloroplatinate	4.49 $\pm 0.06$	6.98 $\pm 0.10$	$2.439 \times 10^6$	$4.066 \times 10^5$	$1.72 \times 10^{-5}$
Creatinine potassium picrate	0.75 $\pm 0.04$	3.02 $\pm 0.16$	$1.746 \times 10^5$	$1.746 \times 10^5$	$1.73 \times 10^{-5}$

\* 32.5 per cent radiomethionine, 67.5 per cent deuteriomethionine.

*Radiocarbon Analyses*—Radioactivity measurements were carried out on the radiomethionine and the choline and creatine derivatives by the procedure described by Melville, Rachele, and Keller (6). Weighed samples of the compounds were burned in a stream of oxygen in a micro combustion tube heated in a furnace at 700–800°. The heated portion of the tube was packed with platinum gauze with a plug of silver gauze at each end. The CO<sub>2</sub> was absorbed in about 6 cc. of 2.5 N NaOH in an absorption tube, the lower half of which was only slightly larger in diameter than the delivery tube, so that the bubbles would be squeezed and rise slowly through the solution (13). A second gas-washing tube containing barium hydroxide followed this in the train. The barium hydroxide remained

clear during the combustions, showing that the NaOH absorbed all the  $\text{CO}_2$ .

The method described by Melville, Rachele, and Keller (6) was used for the precipitation of the barium carbonate. Sodium carbonate was added to the aliquot of the alkaline carbonate solution before the precipitation to bring the thickness of the samples to more than 18 mg. per sq. cm. The  $\text{BaCO}_3$  samples obtained from the three compounds were counted with a thin mica window, bell-shaped, Geiger-Müller counter and scaling circuit. The samples were all of the same area, 2.32 sq. cm., and were placed in the same position with respect to the counter. The samples were counted in rotation until a total of 10,240 counts had been collected from each sample. The probable error of the net counts per minute due to random distribution of the disintegrations was  $\pm 0.7$  per cent (14). The over-all probable error of these radioactivity measurements was estimated to be approximately  $\pm 3$  per cent.

The combustion and precipitation steps in the analysis of the different compounds were carried out at different times and the  $\text{BaCO}_3$  samples were kept in covered Petri dishes until they were counted. Armstrong and Schubert (15) have shown that such samples lose activity if kept in an atmosphere containing both water vapor and  $\text{CO}_2$ . It was therefore important for us to determine whether there was significant loss of activity from our samples on standing.  $\text{BaCO}_3$  samples from a combustion of the radiomethionine were stored for 100 days in a covered Petri dish. A second combustion was then run, and the  $\text{BaCO}_3$  samples from the two combustions were compared. The results of the counting showed that the two analyses agreed within 2 per cent. We therefore concluded that there was no significant loss of activity from our samples stored in this way.

In calculating the radiocarbon content of the compounds the net counts were corrected to  $I_0$ , which is the activity corrected for self-absorption, by using the theoretical self-absorption equation (16, 17). The value of the absorption coefficient,  $\alpha$ , was taken as 0.32 sq. cm. per mg. (18). Two other values have been reported for this absorption coefficient,  $0.285 \pm 0.008$  sq. cm. per mg. and 0.29 sq. cm. per mg. (19). All three of these values for  $\alpha$  have been found to give theoretical curves which fit the experimentally determined values for the self-absorption of the  $\text{C}^{14}$  electrons in  $\text{BaCO}_3$  samples (18, 19).

The corrected counts per minute per millimole of the three compounds are given in column (C) of Table I. The corrected counts per minute per millimole of methyl are given in column (D). These latter values were calculated on the assumption that all the  $\text{C}^{14}$  in the compounds was present in the methyl groups. The activity of the dietary doubly labeled methionine was calculated from the proportion of radiomethionine in it.

Finally, the ratios of deuterium to radiocarbon in the methyl groups of the dietary methionine and the isolated choline and creatine were calculated, and these ratios are recorded in the last column of Table I.

The authors wish to thank Miss Josephine E. Tietzman for the microanalyses reported in this paper.

#### SUMMARY

L-Methionine with the methyl group labeled with  $C^{14}$  and deuterium was fed to a rat for 4 days. The choline and creatine were isolated from the body of the rat and analyzed for both isotopes. Within the limits of experimental error, the ratio of deuterium to  $C^{14}$  in the methyl groups of the choline and creatine was found to be the same as it was in the methyl group of the dietary methionine.

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## FURTHER STUDIES ON LIPIDE STIMULATION OF LACTOBACILLUS CASEI. II

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Investigations of certain lipide materials exhibiting biotin activity for *Lactobacillus casei* have been reported previously (1, 2). These researches have been continued along three somewhat distinct lines: (a) an investigation of microbiologically stimulatory lipides from rice polish in relation to their biotin value for the chick; (b) a study of the lag period observed in the oleic acid and linoleic acid stimulation of *Lactobacillus casei*; and (c) a search for biotin-active substances, lipide in nature but not related to the fatty acids or their esters.

### EXPERIMENTAL

*Chick Growth Study*—Lipide stimulation of *Lactobacillus casei* was first reported from observations made in the routine assay of rice and its milled products (3). It was considered worth while to investigate the biotin value of rice polish, a natural product with high lipide stimulatory value for *Lactobacillus casei*, in the growth of a higher animal such as the chick.

The experiment was designed to use day-old, white Leghorn chicks as hatched, placed in the following groups: Group A, fourteen chicks on a standard basal diet containing added synthetic biotin; Group B, twelve chicks on the standard basal diet containing no biotin; Group C, twelve chicks on the standard basal diet containing whole rice polish as a source of biotin, biotin content being calculated from "stimulated" microbiological assay values; and Group D, twelve chicks on standard basal diet containing whole rice polish as a source of biotin, biotin content being calculated from correct microbiological assay values (filtered extracts). From the outline of the experiment, it may be seen that Group C received only part of the biotin represented by the assay value, since much of that figure represented lipides with biotin activity for the test microorganism. In planning the experiment, it was felt that if the chicks in Group C showed dermatitis, as compared with those in Group D, the stimulation observed in the microbiological assay could not be attributable to substances with similar biotin activity for chicks.

The standard diet selected was that described by Hegsted *et al.* (4) for

the study of biotin requirement of chicks. Sources of specialized materials are given in the original reference. All diets were supplied *ad libitum* and made up fresh every few days as described below.

Variations in the diets were as follows for the four groups outlined above. For Group A, 100  $\gamma$  of biotin were added per kilo of basal diet. Pure crystalline biotin<sup>1</sup> was dissolved in a small quantity of water and an appropriate amount of the resulting solution was poured over the casein. Thorough mixing distributed the vitamin well throughout the casein, which was then mixed with the remainder of the ingredients. 40 drops of haliver oil were added to the required amount of soy bean oil, well mixed, and the oily solution blended with the other ingredients. This quantity of haliver oil was calculated from the daily requirement of the chick and the estimated daily food consumption. For Group B, the diet differed from that for Group A only in the respect that it contained no added biotin. For Group C, 77 gm. of carefully sifted rice polish were incorporated into a kilo of feed and adjustments made for the protein, starch, and oil contained in the rice polish. This quantity of rice polish was calculated to supply 100  $\gamma$  of biotin from the uncorrected (stimulated) assay value of 1.28  $\gamma$  per gm. obtained on unfiltered extracts of the rice polish. The basal diet for Group D was adjusted to include 250 gm. of rice polish per kilo of feed with adjustments in the protein, starch, and oil of the ration. Since the assay value of filtered extracts (lipide-free thereby) of this lot of rice polish was 0.4  $\gamma$  per gm., 250 gm. of polish were required to supply 100  $\gamma$  of biotin. The protein content of rice polish is small enough to permit the substitution described without disturbing the protein quality of the four diets.

The criteria for judging biotin deficiency were selected from the many reports of the symptoms. The scaly dermatitis and perosis characteristics of biotin-deficient chicks have been described by Ansbacher and Landy (5), Jukes and Bird (6), and Richardson, Hogan, and Miller (7). The scaly skin, hemorrhagic fissures of the toes and soles of the feet, lesions at the corners of the beak, as well as perosis were all taken as symptoms of biotin deficiency.

The chicks were banded and weighed every 2 days for 4 weeks. At each weighing, examination was made for deficiency symptoms and all observations recorded. The deficiency and mortality figures are given in Table I.

*Investigation of Fatty Acid Lag Period*—In their studies on lipide stimulation of bacteria, Dubos and Davis (8-10) reported that the growth of *Mycobacterium tuberculosis* was enhanced by a number of long chain fatty acids, provided the acids were first esterified or bound in some way to serum albumin. That particular organism synthesizes its own biotin, and in that

<sup>1</sup> Crystalline biotin was kindly supplied by Merck and Company, Rahway, New Jersey.



respect the phenomenon observed differs somewhat from the case for *Lactobacillus casei*. It has been pointed out previously by this laboratory that free oleic and elaidic acids would permit growth of *Lactobacillus casei* in the absence of biotin, provided sufficient time was allowed to overcome an initial lag, usually 48 hours for oleic acid (1). In view of the findings of Dubos and Davis, it appeared that this lag period might represent time required for the organism to combine the oleic acid with protein in a specific manner, even though the oleic acid is undoubtedly adsorbed rapidly upon the surface of the bacteria, as shown by the fact that oleic acid in the presence of biotin inhibits growth for 48 hours (1).

TABLE I

*Effect of Rice Polish Lipides As Biotin Substitutes in Nutrition of Chick at End of 4 Weeks Growth*

Group	No. of chicks	No. dead from extraneous causes	No. dead from biotin deficiency	No. with dermatitis or lesions	No. with perosis	Total No. dead from or affected by lack of biotin*	Normal chicks
							<i>per cent</i>
A	14	0	1†	0	0	1	93
B	12	1	1	7	4	9	25
C	12	0	0	9	5	10	17
D	12	0	0	0	0	0	100

\* This figure is not necessarily the sum of the three middle columns, since some chicks showed both perosis and dermatitis.

† Although fed a diet containing biotin sufficient to maintain health in other control chicks of Group A, this individual failed to gain weight during the course of the 3rd week and developed such severe perosis that the animal was sacrificed.

It was then decided to add suitable protein, sterile and biotin-free, to biotin-free tubes containing fatty acid, to inoculate with *Lactobacillus casei*, and observe what the effect would be on the usual lag period. Bovine serum albumin was selected as a suitable protein, since it is known to combine rapidly with fatty acids (8, 11). The albumin was added aseptically to the cooled, sterilized tubes of medium.

Two series of comparisons were made, the first with oleic acid and the second with linoleic acid, the latter having a lag period of about 7 days before stimulating growth of *Lactobacillus casei* in biotin-free media. The serum albumin was rendered completely free of biotin by norit and peroxide treatment, but was not denatured in the process. The final product was acceptably sterile, since uninoculated duplicates containing sterile basal medium and the albumin preparation consistently failed to show the growth of any contaminating organisms. No data are given for inoculated

tubes containing only basal medium and albumin. When incubated following inoculation, they supported no growth at all and hence were proved to be biotin-free. The data are presented in Table II.

*Investigations of Non-Fatty Acid Surface-Active Materials*—Proper interpretation of the rapidly accumulating data on lipide stimulation of *Lactobacillus casei* and *Lactobacillus arabinosus* (1, 2, 12-15, to cite only a few)

TABLE II

*Acid Production in Tubes containing Either Biotin, Fatty Acid, or Fatty Acid Plus Albumin*

Series	Hrs. after inoculation	0.1 N acid per tube*			
		No biotin added	1000 micro-micrograms biotin per tube	100 $\gamma$ fatty acid per tube	100 $\gamma$ fatty acid + 20 mg. albumin per tube
		ml.	ml.	ml.	ml.
Oleic acid	16.7	2.30	2.71	2.21	2.74
	19.5	2.21	3.21	2.13	3.51
	23.5	2.25	4.36	2.21	5.87
	27.8	2.52	5.80	2.18	7.54
	40.7	2.52	10.55	2.43	12.10
	43.2	2.78	11.44	2.58	12.92
	51.2	3.26	13.23	4.02	14.40
	64.8		16.03	7.44	16.53
Linoleic acid	17.0	2.18	2.19	2.20	2.47
	19.5	2.20	2.40	2.08	3.18
	23.0	2.22	3.16	2.06	4.50
	29.0	2.37	5.24	2.10	7.13
	42.0	2.49	9.75	2.10	11.39
	64.0	2.96	15.29	2.35	14.86
	136.0	2.48	18.88	4.05	17.55
	162.0		19.32	7.98	17.64

\* Each figure represents the average of two tubes from a group of replicates inoculated simultaneously.

rests largely on determining the specificity of the effect for fatty acids. The surface-active nature of biotin (16) and of the lipides with known biotin activity suggests an examination of other surface-active materials, especially those not chemically related to the fatty acids or their esters. Various concentrations of these materials were added to the basal medium, and were tested for biotin activity in the absence of biotin, or for inhibitory effects in the presence of biotin. The tube contents were titrated at the end of a 72 hour incubation period and compared with standards.

## DISCUSSION

From Table I it may be seen that chicks fed the complete diet containing pure biotin (Group A) and those fed the same ration with biotin supplied by rice polish in amounts calculated from microbiological assay of lipide-free samples (Group D) were on the whole free from symptoms of biotin deficiency. On the other hand, only 25 per cent of those chicks receiving the biotin-free basal diet (Group B) were normal at the end of 4 weeks. In Group C, chicks receiving rice polish as a source of biotin with quantities calculated from assay of samples containing lipides stimulatory to *Lactobacillus casei*, the percentage of chicks found normal at the end of 4 weeks

TABLE III  
*Biotin Activity of Non-Fatty Acid Surface-Active Materials*

Surface-active agent	Per tube	Effect in biotin-free tubes	Effect in tubes containing 1000 micrograms biotin
Nopalcol 6-benzoate,* non-ionic ethylene oxide ester	50-1,000	No stimulation	No inhibition
Nopalcol 6-abietate,* non-ionic ethylene oxide ester	50-5,000	"	"
Nopalcol 6-naphthenate,* non-ionic ethylene oxide ester	50-5,000	"	"
Aerosol OT, dioctyl ester of sodium sulfosuccinic acid	20-10,000	"	
Cholesterol, recrystallized	100-400	"	

\* Supplied through the courtesy of the Nopco Chemical Company, Harrison, New Jersey.

was even lower than that for Group B. From these data it is evident that the lipides stimulatory to *Lactobacillus casei* and responsible for the apparent high biotin content of unfiltered extracts used for microbiological assay were unable to perform the same function when supplied as part of the biotin needed by the chicks.

In examining the albumin-fatty acid data, it may be noted in Table II that the presence of albumin not only obviated the lag period for oleic and linoleic acids but in the former case brought about growth at a rate exceeding that in the tubes containing biotin, somewhat after the manner observed previously for the non-ionic detergents (2). It appears then that the lag period may represent time necessary for proper orientation of lipoprotein combination, since the addition of suitable protein (or the introduction of

the fatty acid in a non-ionic ester referred to above) permits immediate growth in biotin-free media.

With regard to the non-fatty acid detergents examined thus far and reported in Table III, none has been found to stimulate the growth of *Lactobacillus casei* or show biotin activity. These findings do not preclude the possibility that such materials exist, but the stimulatory effects observed thus far are limited to the higher fatty acids or their derivatives.

#### SUMMARY

Rice polish lipides having biotin activity for *Lactobacillus casei* and *Lactobacillus arabinosus* did not substitute for biotin in the nutrition of the white Leghorn chick.

The lag periods previously observed in the growth stimulation of *Lactobacillus casei* by oleic acid and linoleic acid completely disappeared on the addition of sterile, biotin-free, bovine serum albumin to the biotin-free culture media containing the fatty acids. In the early stages of growth of the microorganism, the rate exceeded that observed in the tubes containing biotin.

Of the non-fatty acid surface-active agents thus examined, several of which are non-ionic and non-inhibitory, none has been found which will stimulate the growth of *Lactobacillus casei* in the absence of biotin.

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## SURFACE ACTIVITY OF BIOTIN

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(Received for publication, June 18, 1948)

Certain surface-active materials such as oleic acid and its esters substitute readily for biotin in the synthetic medium used for culturing *Lactobacillus casei* (1). (This medium contains ample aspartate, the biotin-aspartate relationship being now established (2).) How these surface-active materials, principally oleates, permit the bacteria to dispense with biotin is at present uncertain, although it has been suggested by Snell and others (3, 4) that biotin is essential for the synthesis of oleic acid and therefore is not necessary when oleic acid is supplied. A different line of approach makes the assumption that oleic acid and similar surface-active materials substitute for biotin and that their activity can be explained on the basis of their physicochemical behavior. A comparison of the surface behavior of biotin with such physiologically active materials should prove illuminating to the problem.

To investigate the surface activity of biotin, a study has been made of the electrophoretic mobility of *Lactobacillus casei* in the presence of increasing concentrations of (a) biotin, (b) oleic acid, and (c) anionic, cationic, and non-ionic detergents. Supplementary to the cataphoretic study, the surface activity of biotin was investigated polarographically, a comparison being made between biotin and a substance of known surface-active nature.

### EXPERIMENTAL

*Electrophoresis Studies*—Electrophoresis measurements were made in an Abramson cell by employing the standard method for electrophoretic investigation described by Moyer (5). Cultures of *Lactobacillus casei* were grown in yeast extract-glucose-sodium acetate broth for 20 to 24 hours, after which time they were centrifuged and washed three times in buffer of desired pH and of ionic strength 0.02. The buffered solutions of surface-active materials were inoculated with a suspension of the bacteria and allowed to stand 30 minutes before reading.

Acetate and phosphate buffers of ionic strength 0.02 were used throughout the study. The synthetic detergents used were: Nopco 2173-B, a cationic oleic amine sulfate; Nopco 1285, an anionic sulfated butyl oleate; and Nopalcol 6-O, a non-ionic oleate. These surface-active agents were

generously supplied by Nopco Chemical Company, Harrison, New Jersey. The Nopalcol 6-O is strongly stimulatory to *Lactobacillus casei*, but the other two products produce no stimulation in the absence of biotin and are highly inhibitory in the presence of biotin.<sup>1</sup> A fine emulsion of oleic acid in water was used since the fatty acid is practically insoluble in water. The concentrations of the surface-active agents used had little effect upon the ionic strength of the buffered solutions and had no effect on the conductivities.

*Polarographic Studies*—An American model XI Heyrovsky polarograph was used for recording the polarograms. Solutions to be polarographed were purged of dissolved oxygen by bubbling oxygen-free nitrogen through them. Concentrations of copper ion, surface-active materials, and supporting electrolyte are given in the legend of Fig. 3. The applied potential range for each curve was 0.0000 to 0.9000 volt and the pH of the copper solutions was 5.5.

#### DISCUSSION

Fig. 1 shows the effect of cationic and anionic detergents on the electrophoretic mobility of *Lactobacillus casei*. The anionic detergent, Nopco 1285, had no effect on the mobility of the organism over the concentration range studied. It would appear, then, either that the detergent was not adsorbed or that the amount adsorbed did not affect the charge on the bacteria. Since Nopco 1285 at concentrations of  $10^{-3}$  to  $10^{-6}$  M is highly inhibitory to *L. casei*, it would seem logical to believe that some of the detergent must be adsorbed in order to interfere with the metabolism of the organisms. It may be that the amounts adsorbed, though influencing the growth of the bacteria, are too small to affect the surface charge. The effect of Nopco 2173-B, cationic, on the mobility of *L. casei* was typical of the general effect of cationic detergents on the electrophoretic mobility of bacteria, as shown by Dyar and Ordal (6) in their comprehensive study on the electrokinetics of bacterial surfaces. The concentration at which a reversal of charge occurred was quite easily reproduceable and represents the point at which the negative charge of the bacterial protein surface is reduced to zero by the adsorption of cations of the detergent. Nopco 2173-B, however, is strongly inhibitory to *L. casei* over the entire concentration range shown in Fig. 1, indicating that the reversal of charge is not necessary to bacteriostasis. According to the argument advanced by Dyar and Ordal, the effect of these two substances indicates that the surface of *L. casei* must be principally protein in nature, especially since the anionic detergent did not alter the mobility. Another anionic detergent, Nopcocas-

<sup>1</sup> Williams, V. R., unpublished data.

tor, for which the data are not given, produced the same type of mobility-concentration curve as did Nopco 1285.

In Fig. 2 is seen the influence of biotin and two substances with biotin activity, oleic acid and Nopalcol 6-O, upon the mobility of *Lactobacillus casei*. The state of ionization was more similar than might be realized on first inspection. The Nopalcol 6-O is freely water-soluble, but non-ionic; oleic acid as used was non-ionized, since it is practically insoluble in water and therefore was used as a fine emulsion of droplets in water; biotin

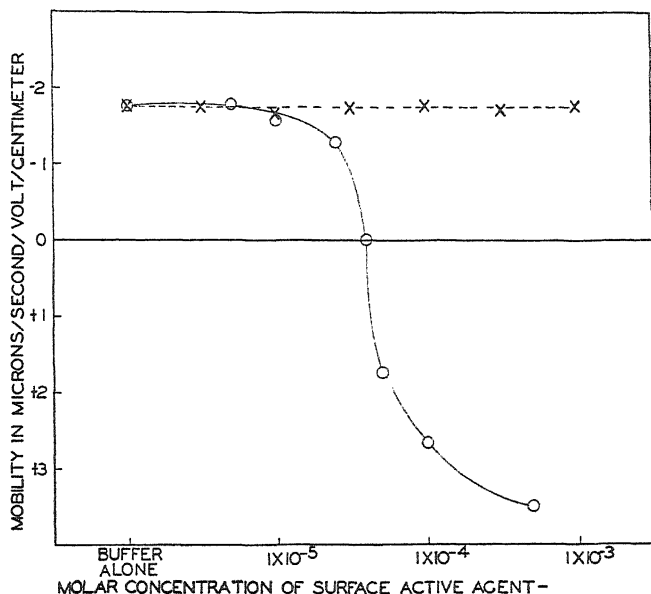


FIG. 1. Mobility of *Lactobacillus casei* in presence of ionic detergents. X, anionic detergent, Nopco 1285, at pH 6.8; O, cationic detergent, Nopco 2173-B, at pH 6.8.

likewise is relatively insoluble in water and has a very low ionization constant.<sup>1</sup> Fig. 2 shows that these three substances had a like effect on the mobility of *L. casei*, quite different from that of either the anionic or the cationic detergent (Fig. 1). In each case, a small but definite decrease was observed in the mobility. Since the changes resulting from increased concentrations of biotin were not so marked as those produced by oleic acid or Nopalcol 6-O, the biotin data were subjected to statistical analysis to evaluate the significance of the mobility decreases. As is shown in Table I, "t" values were calculated, comparing the mobility obtained with the lowest concentration of biotin ( $5 \times 10^{-7}$  M) with those obtained for the higher concentrations. The mobility of the bacteria in the lowest

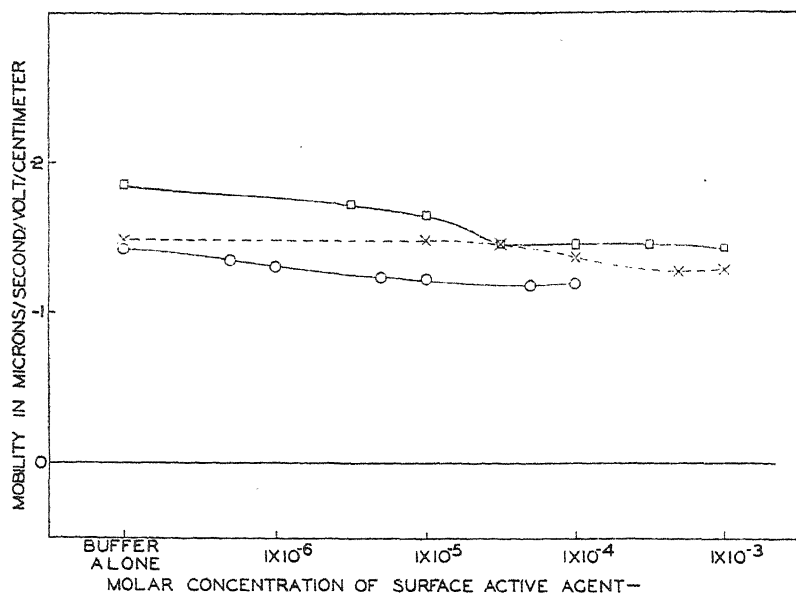


FIG. 2. Mobility-concentration curves for *Lactobacillus casei*. □, non-ionic detergent, Nopalcol 6-O, at pH 6.8; X, oleic acid, at pH 5.6; O, biotin, at pH 5.2.

TABLE I  
Electrophoretic Mobility of Bacteria at Varying Biotin Concentrations

Sample No.	Biotin concentration	No. of observations	Mobility $\pm$ standard error	Mean difference $\pm$ standard error of difference*	<i>t</i> *
	moles per l.		$\mu$ per sec. per volt per cm.		
1	0 (buffer only)	16	1.415 $\pm$ 0.014		
2	5 $\times$ 10 <sup>-7</sup>	16	1.354 $\pm$ 0.015		
3	1 $\times$ 10 <sup>-6</sup>	10	1.311 $\pm$ 0.053	0.043 $\pm$ 0.023	1.9
4	5 $\times$ 10 <sup>-6</sup>	12	1.230 $\pm$ 0.017	0.124 $\pm$ 0.023	5.4
5	1 $\times$ 10 <sup>-5</sup>	20	1.234 $\pm$ 0.018	0.120 $\pm$ 0.024	5.0
6	5 $\times$ 10 <sup>-5</sup>	16	1.181 $\pm$ 0.010	0.173 $\pm$ 0.018	9.6
7	1 $\times$ 10 <sup>-4</sup>	22	1.188 $\pm$ 0.013	0.166 $\pm$ 0.020	8.3

\* *t* values calculated for comparisons of corresponding concentrations with 5  $\times$  10<sup>-7</sup> M.

concentration of biotin was used as a reference point rather than mobility in buffer alone, since it was felt that such a calculation would be more critical. With the exception of the 1  $\times$  10<sup>-6</sup> M concentration, all differences observed were found to be significant, those obtained with the two highest concentrations particularly.



From the above curves (Fig. 2) and the data analysis (Table I), it is apparent that the substances examined were adsorbed upon the surface of the bacteria, since reduction in charge was observed. The effect, however, was unlike that of the cationic detergent, since stabilization occurred without reversal of charge. If it is assumed that all the substances shown in Fig. 2 were non-ionic, adsorption must have been due to attraction for the surface-active agent as a dipole or to a solubility effect. A solubility effect would depend upon appreciable amounts of lipide in the cell surface. The behavior of Nopco 1285 precludes this deduction, according to arguments cited previously (6). Possibly, then, adsorption takes place because of attraction of dipoles or induced dipoles.

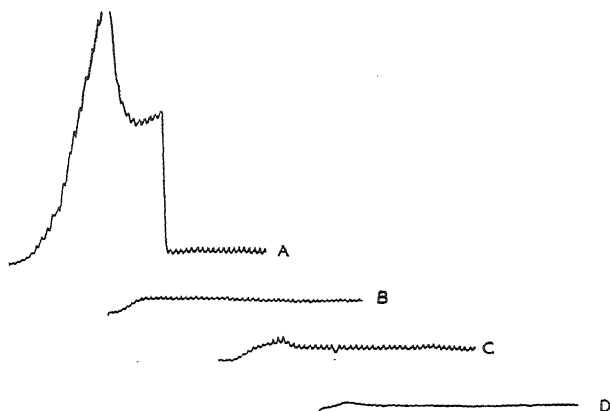


FIG. 3. Effect of biotin on copper maximum. Curve A, 0.002 M  $\text{Cu}^{++}$  in 0.1 M KCl; Curve B, 0.002 M  $\text{Cu}^{++}$  and 0.01 per cent gelatin in 0.1 M KCl; Curve C, 0.002 M  $\text{Cu}^{++}$  and 0.01 per cent biotin in 0.1 M KCl; Curve D, 0.01 per cent biotin in 0.1 M KCl.

The main problem is the decrease in charge, and several hypotheses suggest themselves. The layer of surface-active substance between the cell surface and surrounding medium may have affected the viscous flow of the bacterial cell when moving under the influence of an electrical potential. Another possibility is that the adsorbed layer of surface-active material may have altered the dimensions of the double layer so as to effect a charge decrease. Whatever the explanation is, the similarity in behavior of these three substances points to the surface activity of biotin, and, though it cannot be said that these findings explain the biotin activity of oleic acid and Nopalcol 6-O, the results invite speculation.

It should be pointed out that, as in the case of Nopco 2173-B (Fig. 1), the concentrations of oleic acid, Nopalcol 6-O, and biotin necessary to produce significant decreases in mobility were higher than those sufficient to maintain growth of *Lactobacillus casei* (7, 1). This fact in no way

invalidates the evidence for surface activity as seen in the electrophoretic data, but means simply that the changes produced by lower concentrations were too small to measure. Theoretical development of this point must await additional information regarding the amount of biotin actually adsorbed onto the surface of the bacteria and molecular orientation in the monolayer.

Fig. 3 confirms the indications of surface activity for biotin observed in Fig. 2. Curve A is the normal copper wave; Curve B shows the inhibition of the copper maximum in the presence of a substance of known surface activity, gelatin. Curve C for copper and biotin shows that the biotin behaves similarly to the gelatin in inhibiting the copper maximum, only a suggestion of a maximum being evident. Curve D gives the wave for biotin alone and shows that, over the potential range examined, biotin gave no evidence of an oxidation-reduction reaction. This method for detecting surface activity is fairly well recognized and has been used by Veldstra (8).

#### SUMMARY

Electrophoretic examination of the mobility of *Lactobacillus casei* in the presence of increasing concentrations of anionic and cationic detergents showed the former to have no effect on the mobility, whereas the latter produced a decrease, reversal, and finally stabilization of charge. In the presence of Nopalcol 6-O, a non-ionic detergent, and oleic acid and biotin, both of which were essentially non-ionized, a decrease in mobility followed by stabilization was observed. These findings were accepted as evidence that the substances were adsorbed upon the surface of the bacteria, and indicated a surface-active nature for biotin. The surface activity of biotin was confirmed by polarographic study of its effect on the copper wave.

The authors express their thanks to Dr. Philip W. West and Mr. Ernest J. Breda for their help in the polarographic determinations.

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# DETERMINATION OF SERUM PROTEINS BY MEANS OF THE BIURET REACTION

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(Received for publication, August 2, 1948)

In the course of an investigation of the biochemical changes following experimental liver injury we felt the need of a simple, rapid, and accurate method for determining the protein fractions in small amounts of serum. Among the simpler procedures known, the biuret reaction seemed to offer the most encouraging possibilities.

Variations and improvements in the application of the biuret reaction to clinical chemistry can be traced in the works of Autenrieth (1), Hiller (2), Fine (3), Kingsley (4), and Robinson and Hogden (5). Kingsley (6) simplified the technique by adding serum directly to a "one piece" reagent. Efforts have been made to increase the stability of such biuret reagents with ethylene glycol (7), tartrate (8), and citrate (9).<sup>1</sup>

We began our investigation with Kingsley's (6) method and report briefly on the two main difficulties encountered in its use. The first is that the total protein (TP) reagent and, to a lesser extent, the albumin (ALB) reagent are not sufficiently stable. The length of time they remain so depends upon the technique of their preparation. One consequence of this variable stability is a difficulty in duplicating calibration curves with different lots of reagent. Errors may arise when results with a new reagent are read from an old calibration curve. Serious errors occur if a reagent is used after the separation of any black deposit gives evidence of deterioration.

A second difficulty has been that total protein estimations made with the TP reagent and read, as prescribed, from calibration curves prepared with the ALB reagent have tended to be too low. Recorded in Table I are the results of a number of analyses in which Kingsley's biuret procedure has been compared with the Kjeldahl method<sup>2</sup> on both normal and ab-

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<sup>1</sup> We have had access only to an abstract of this paper. Even with 10 times the amount of citrate there stated the reagent has been found unstable.

<sup>2</sup> Kjeldahl digestion with copper selenite catalyst (10) heated 15 to 20 minutes after clearing. Distillation into boric acid and titration with standard acid (11); protein = nitrogen (corrected for non-protein nitrogen)  $\times$  6.25. Values so obtained were 99+ per cent of the results with a digestion time of 3 hours or longer.

normal human and dog sera. It will be noted that, in such a comparison, the results for total protein by Kingsley's method are invariably and considerably low, while the results for albumin are in reasonably good agreement. Kingsley (12) reported low values for total protein by his method in patients suffering from chronic liver disease, but good agreement with the Kjeldahl method in normal individuals or persons suffering from a variety of other diseases. We have noted the discrepancy in any serum when a result for total protein with the TP reagent is read from a calibration curve prepared with the ALB reagent. The error, it is felt, must be

TABLE I

*Results of Comparison of Analyses by Kingsley and Kjeldahl Methods*

The values are measured in gm. per cent.

Serum		Diagnosis	Total protein			Albumin	
			Kjeldahl nitrogen	Kingsley biuret Total protein reagent	Albumin reagent	Kjeldahl nitrogen	Kingsley biuret
Human	A. G.	Normal	7.49	7.10	7.45		
	C. B.	"	7.01	6.60	7.10		
	B. B.	"	7.04	6.50	7.10		
	J. D.	"	6.70	6.30	6.65		
	McK.	Lupus erythematosus	7.38	6.30		3.45	3.04
	M. S.	Cirrhosis	6.00	5.00		3.17	3.07
Dog No.	1	Normal	6.32	5.92			
	2	"	6.55	6.05		3.68	3.36
	5	"	6.36	6.17		3.94	4.00
	14	"	5.95	5.30			
	15	"	6.34	5.95			
	1	CCl <sub>4</sub> liver injury	5.40	5.00	5.53	3.26	3.52
	10	" " "	6.32	5.92	6.30	3.38	3.22

attributed to differences in behavior of the two reagents, prepared and stored at different strengths, one diluted to the strength of the other only at the time of analysis. Note, for example, that in Table I, sixth column, where total protein is determined by adding diluted serum to the ALB reagent, agreement with the Kjeldahl determination is satisfactory.

Our experience with Mehl's (7) method has been more limited. The reagent is stable but not easily duplicated, especially if the glycol contains any reducing substances as impurity. Certain minor disadvantages of the method are not encountered in the procedures which follow.

Weichselbaum (8) has described a biuret reagent stabilized with sodium potassium tartrate and potassium iodide. It is characterized by its high

content of copper and low concentration of alkali. When a biuret reaction is developed with protein, the change appears to be simply an intensification of the blue color of the reagent, though spectrophotometric analysis reveals the presence of the reddish violet complex with an absorption maximum at about  $5.45 \mu$ . In a foot-note, this author describes a second, "dilute" reagent as having the advantage of added sensitivity when the analyst is using a spectrophotometer or photoelectric colorimeter. This reagent has a fifth as much copper, and the reddish violet color developed upon addition of protein is clearly apparent.

It has been our experience that the biuret reagents of Weichselbaum possess the advantages claimed by their originator with respect to stability and optical clarity when mixed with clear sera. Differences between the two reagents, objections to the first, and a paucity of information concerning the behavior of either led us, however, to reinvestigate in some detail the factors affecting optimal sensitivity, stability, and practical usefulness of biuret reagents stabilized with tartrate. Our experiments, which have led to certain modifications in the reagent and in its application to the determination of protein fractions in serum or plasma, are described in the following section.

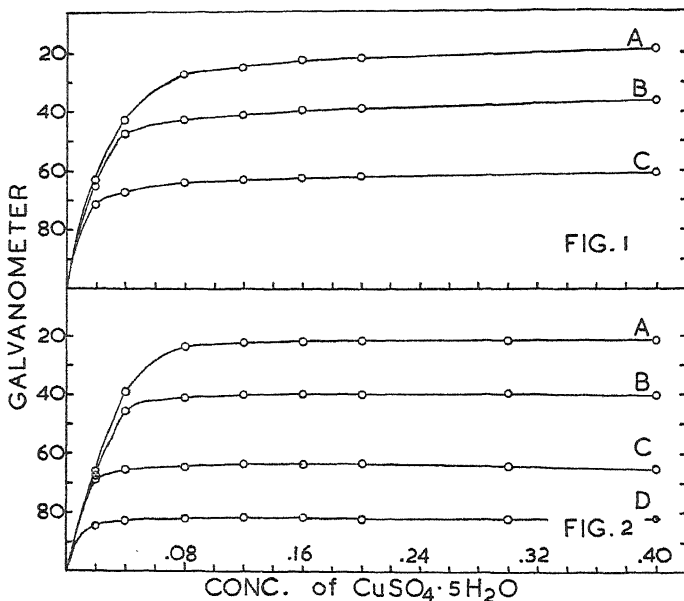
#### *Study of Biuret Reagents Stabilized with Tartrate*

*Concentration of Copper*—The concentration of cupric sulfate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) in the final biuret reaction mixtures of different investigators has varied from about 0.06 to 0.75 gm. per 100 ml., most commonly in the case of serum protein estimations, from 0.1 to 0.2 per cent. Mehl has related the optical density of reaction mixtures to copper concentration for a reagent stabilized with ethylene glycol. Comparable data for a biuret reagent containing tartrate are reported in Figs. 1 and 2.

It will be noted in both Figs. 1 and 2 that, at the protein levels stated, color development increases with increasing concentrations of copper sulfate, rapidly at first, then more slowly, and finally (Fig. 1) reaches a stage in which further additions of copper effect only a very slight, uniform, and linear increase in optical density of the copper-protein complex. This last rise in the curve has been found to result from the change in tartrate to copper ratio, because (Fig. 2), when this ratio is held constant, the curve becomes essentially horizontal over a wide range of copper concentrations. It will be noted that at protein levels up to 0.04 per cent (4 per cent in the undiluted serum) a reagent yielding a final copper sulfate concentration of 0.06 per cent would be adequate. For sera containing about 7 per cent protein, the reaction mixture should contain not less than 0.09 per cent copper sulfate and, for levels up to 12 per cent protein, a concentration of 0.12 per cent is desirable. With the serum dilution used,

an increase in copper above this level makes no apparent contribution to the biuret reaction. Its chief effect is the somewhat undesirable one of increasing the optical density of the reagent "blank."

*Concentration of Sodium Hydroxide*—The importance of sodium hydroxide in the biuret reaction has long been recognized and was studied in some detail by Rising and Johnson (13). Before the development of stabilized reagents, the biuret reaction was generally carried out in a



FIGS. 1 AND 2. Effect of copper sulfate concentration upon color development in biuret reaction mixtures containing 2.4 per cent sodium hydroxide and protein concentrations of Curve A, 0.127 per cent; Curve B, 0.077 per cent; Curve C, 0.039 per cent, and Curve D, 0.016 per cent. In Fig. 1, the sodium potassium tartrate concentration is constant at 0.48 per cent. In Fig. 2, the tartrate concentration is in each case 3.5 times that of the copper sulfate present.

medium containing about 3 per cent alkali. It was necessary to separate a precipitate of cupric hydroxide before color comparisons could be made. Kingsley (6) found that very high concentrations of alkali (12 to 17 per cent) prevented cupric hydroxide precipitation and by this means obtained fairly stable "one piece" biuret reagents. With the introduction of reagents containing glycol or tartrate, the need for such high levels of sodium hydroxide was obviated. Mehl obtained best results with 2 to 4 per cent alkali in the presence of glycol. Weichselbaum concluded that 0.4 per cent sodium hydroxide was optimal for reagents containing tartrate.

We have investigated the effect of alkali concentration in a reaction mixture containing 0.12 per cent copper sulfate and 0.48 per cent sodium potassium tartrate and observed the color development shown in Fig. 3.

It will be noted that with 0.2 per cent alkali color development is poor, but that from a minimum of 0.4 per cent to about 7 per cent the results

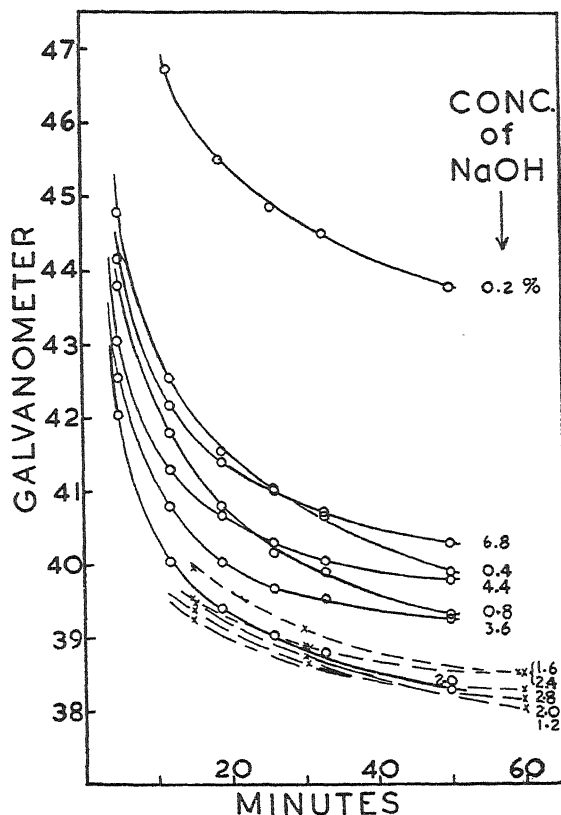


FIG. 3. The effect of sodium hydroxide upon color development in biuret mixtures containing 0.12 per cent copper sulfate, 0.48 per cent sodium potassium tartrate, and 0.077 per cent protein.

are not markedly different. With the final copper sulfate and tartrate concentrations here employed, it would appear that optimal biuret color development occurs with about 1.2 to 2.8 per cent alkali. It has been observed also that an increase in copper concentration tends to shift this optimal alkalinity toward higher values. From the shape of the curves shown it can be noted that the higher alkali concentrations favor a somewhat more rapid development of maximal color.

With low concentrations of alkali, a reaction mixture will remain clear for several days. When levels above about 2 per cent are used, the mixture may show, but only after 12 to 24 hours, a slight flocculent coagulum. This precipitation, which occurs when alkali alone is added to serum, has no significance for readings made at 30 minutes.

*Effects of Tartrate*—The use of sodium potassium tartrate as a stabilizing agent in the copper-containing reagents for the determination of reducing sugars has been known for many years. Shaffer and Somogyi (14) found that an amount of tartrate equal to about 3 times the weight of copper sulfate was most satisfactory in their reagent which contained approximately 3 per cent alkali. Weichselbaum employs triple amounts of tartrate in both of the low alkali biuret reagents which he has described.

We confirm the observation that a minimal 3:1 ratio of tartrate to copper sulfate is necessary if a biuret reagent containing moderate amounts of alkali is to keep longer than a few weeks. Having established a minimal copper sulfate concentration of about 0.1 per cent, it follows that the reagent must contain 0.3 per cent or more of sodium potassium tartrate. The addition of such amounts of tartrate causes a very slight retardation of, but a rather distinct diminution in the degree of, color development when protein is added to the reagent (Fig. 4). The presence of tartrate does make it advisable to wait somewhat longer before recording the optical density of the reaction mixture.

Weichselbaum felt it necessary to carry out the reaction in a warm water bath. In Fig. 4 there is also recorded, with our modified biuret reagent, the effect of developing and reading the color reaction at different temperatures. It will be noted that the effect is very slight after 30 minutes, amounting to about 1 scale division for every 10° rise in temperature. It would seem quite satisfactory therefore to develop the reaction at room temperature, within, say, a 5° range. This avoids entirely the complication of heating and, if the color intensity is read at 30 minutes, the changes are so slow that strict timing is not essential.

The inhibiting effect of tartrate is illustrated further in Fig. 5. Here we record, for different concentrations of copper sulfate and a constant amount of sodium hydroxide, the optical density obtained after 30 minutes in the presence of increasing ratios of sodium potassium tartrate to copper sulfate. It will be noted, first, that the greater the concentration of tartrate, for any fixed level of copper, the less the color development when protein is added to that reagent. A comparison of identical tartrate to copper ratios, for different copper levels, shows that the readings are virtually the same. One can conclude, then, that the effect of tartrate depends not on its actual concentration, but upon the relative amounts of tartrate and copper.



It will already be clear that the behavior of a biuret reagent is affected by its content of copper, alkali, and tartrate. An attempt is made to illustrate the interrelationships of all three of these factors in Fig. 6. Here a comparison is made of the color developed upon addition of serum to reagents containing two levels of copper, each with 3 and 10 times the

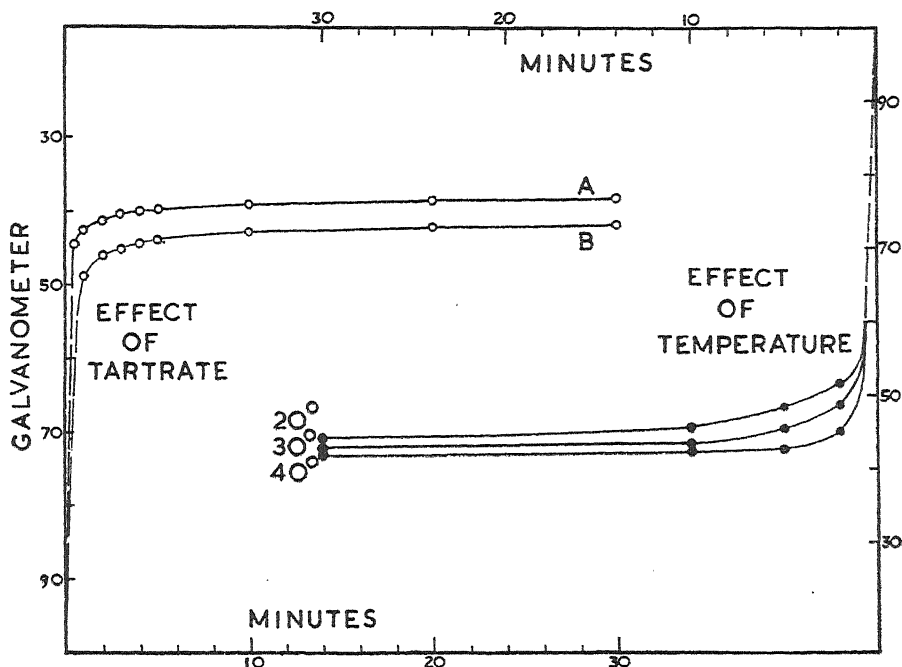


FIG. 4. Rate and degree of color development in biuret reaction mixtures. ○, Curve A, without tartrate, Curve B, with 0.48 per cent sodium potassium tartrate; sodium hydroxide 6 per cent; protein 0.065 per cent. ●, the effect of temperature on a reaction mixture containing 0.12 per cent copper sulfate, 0.48 per cent tartrate, and 2.4 per cent alkali; protein 0.063 per cent.

amount of tartrate, and all four at alkali levels of 0.4, 2.4, and 4.8 per cent. All concentrations shown are those in the final reaction mixture.

From a consideration of Fig. 6 the following statements can be made: (1) In all cases the optical density is greater with the lower tartrate to copper ratio. (2) At the 0.4 per cent alkali level, 0.12 per cent copper sulfate is better than 0.32 per cent for either tartrate ratio. (3) With 2.0 to 2.5 per cent sodium hydroxide, there is no difference between copper levels of 0.12 and 0.32 per cent at a 3:1 tartrate ratio, but 0.12 per cent copper is still somewhat better for a 10:1 ratio. (4) When the alkali concentration is about 5 per cent, the reagent with 0.32 per cent copper

is somewhat better for a 3:1 tartrate ratio, but 0.12 and 0.32 per cent copper sulfate are virtually identical in reagents containing 10 times these amounts of tartrate.

Another important property of biuret reagents stabilized with tartrate is the absence of any serious tendency to develop turbidity in the reaction mixture following the addition of clear serum or plasma. Thus one of the main difficulties of Kingsley's reagent, and a concern with Mehl's procedure, is avoided. Clouding will occur in situations in which the protein concentration is excessively high in relation to the copper content

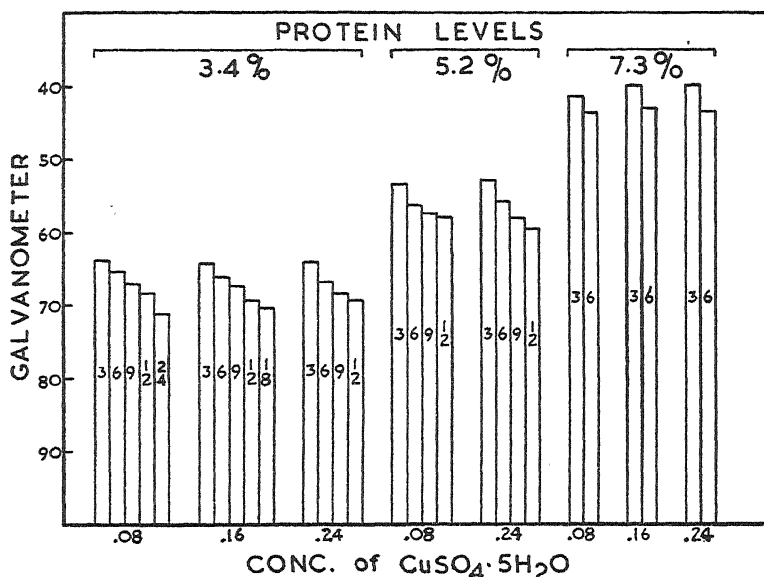


FIG. 5. Effect of the tartrate to copper sulfate ratio, indicated within the columns, on the degree of color development. Serum protein levels and final copper concentrations as shown. Sodium hydroxide 2.4 per cent.

of the reagent. We feel safe, however, in asserting that turbidity will not arise at final 1 in 100 dilutions of sera containing up to 15 per cent protein when the copper sulfate level is 0.12 per cent and the tartrate ratio 4:1. Tartrate is without effect on the precipitate produced by 3 per cent alkali after 12 to 24 hours.

*Use of Potassium Iodide*—Shaffer and Somogyi observed that, in tartrate-containing copper reagents for the determination of reducing sugar, potassium iodide served to prevent "autoreduction" and separation of cuprous oxide. At room temperature, deterioration of the reagent was prevented by 1 gm. of potassium iodide per liter. For maximal stability

during periods of heating, however, it was recommended that 5 gm. per liter be added. Weichselbaum has incorporated 5 gm. of potassium iodide per liter in his reagents.

We have studied the effect of omitting potassium iodide entirely and, with more than 100 different preparations of the reagent, have encountered cuprous oxide precipitation in only one instance. This reduction, and

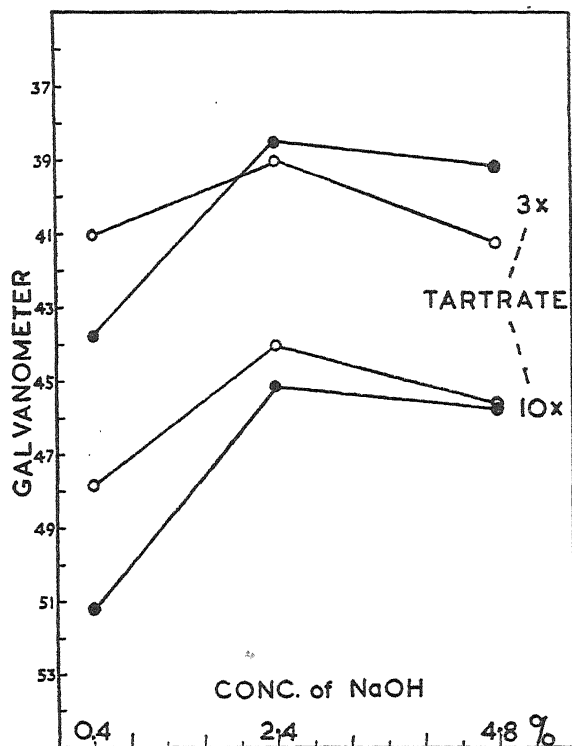


FIG. 6. The interrelationships of copper, tartrate, and alkali concentrations in the biuret reagent. O, 0.12 per cent copper sulfate, ●, 0.32 per cent copper sulfate.

indeed the so called autoreduction, we feel bound to regard as due to some contamination, or impurities in the chemicals used. It is well known that potassium iodide favors the reoxidation of reduced copper and may therefore mask the presence of such impurities. It would seem that potassium iodide can, with reasonable safety, be omitted from the reagent. If, under conditions of preparation or use, any tendency to reduction should be noted, 1 gm. of potassium iodide can be included in each liter of reagent. The presence of this amount of iodide has no detectable effect upon the rate, degree, or quality of biuret color production.

*Choice of Biuret Reagent*—It is plain that there are many biuret reagents, differing slightly in composition, which will work with comparable accuracy and satisfaction if they meet the conditions defined above. The data presented have led us to prefer a reagent containing 0.15 per cent copper sulfate, 0.6 per cent sodium potassium tartrate, and 3.0 per cent sodium hydroxide (with 0.1 per cent potassium iodide optional). When this reagent is mixed in a proportion of 8:2 with 1 in 20 dilutions of serum, the final reaction mixture contains four-fifths of the above concentrations. Such a reagent works exceedingly well in the Evelyn photoelectric and certain other colorimeters. With instruments that are somewhat less sensitive, we recommend that 3 ml. of diluted protein solution be used with 7 ml. of a reagent containing 0.25 per cent copper sulfate, 1.0 per cent sodium potassium tartrate, and 3.5 per cent sodium hydroxide. If readings are made in a single cuvette requiring 10 ml. of solution, the volume of reagent can be increased to allow 2 to 3 ml. of fluid for rinsing.

#### *Determination of Serum Total Protein, Albumin, and Globulins*

An adaptation of the biuret method to the estimation of protein fractions, separated by Kingsley's (6) modification of Howe's procedure (but at a 1 in 20 dilution), is described in detail. The same general technique can be applied to protein fractionations by the sulfite method of Campbell and Hanna (15), the alcohol method of Pillemer and Hutchinsor (16), or the modified sulfate method of Majoor (17).

#### *Reagents—*

*Biuret reagent.* Weigh 1.50 gm. of cupric sulfate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) and 6.0 gm. of sodium potassium tartrate ( $\text{NaKC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$ ); transfer to a dry 1 liter volumetric flask, and dissolve in about 500 ml. of water. Add with constant swirling 300 ml. of 10 per cent sodium hydroxide (prepared from stock, carbonate-free, 65 to 75 per cent sodium hydroxide solution). Make to volume with water, mix, and store in a paraffin-lined bottle. This reagent should keep indefinitely but must be discarded if, as a result of contamination or faulty preparation, it shows signs of depositing any black or reddish precipitate.

*Globulin precipitants.* (a) Sodium sulfate, 22.6 per cent; (b) ethyl ether. Sodium chloride, 0.9 per cent.

#### *Procedure*

- ✓ Select three colorimeter cuvettes (or test-tubes) and mark them Cuvettes B (blank), T (total protein), and A (albumin).

Into Cuvette B pipette 2.0 ml. of 22.6 per cent sodium sulfate solution. This "blank" will serve for all the protein analyses being carried on at any one time.

Into a centrifuge tube measure, with an Ostwald pipette, 0.5 ml. of serum and add 9.5 ml. of 22.6 per cent sodium sulfate. Stopper the tube and mix thoroughly by inversion (not by shaking). *At once* transfer 2.0 ml. of the mixture to Cuvette T.

To the remaining serum-sulfate mixture add 3 ml. of ether, mix for 30 seconds, cap, and centrifuge. Slant the tube and transfer 2.0 ml. of the aqueous phase to Cuvette A.

Now into each of the three cuvettes pipette 8.0 ml. of biuret reagent and mix thoroughly by swirling. Allow these to stand for 30 minutes at room temperature (20–25°).

Using a photoelectric colorimeter, or spectrophotometer, transmitting maximally at 540  $m\mu$ , adjust it to 100 per cent transmission with the "blank" Cuvette B in position. Replace Cuvette B with Cuvettes T and A in turn and record the percentage transmission (or optical density) of each.

Obtain the concentration of total protein and of albumin in the serum by reference to the calibration curve; the total protein minus the albumin gives the globulin concentration.

*Calibration Curve*—Pipette 5.0 ml. of clear, normal serum into a stoppered, graduated cylinder, dilute to 50 ml. with 0.9 per cent sodium chloride, and mix.

Prepare in duplicate a series of nine cuvettes (or test-tubes) and into them pipette successively 2.0, 1.85, 1.70, 1.55, 1.40, 1.20, 1.00, 0.80, and 0.60 ml. of 0.9 per cent saline. Then, in the same order, add 0, 0.15, 0.30, 0.45, 0.60, 0.80, 1.00, 1.20, and 1.40 ml. of the diluted serum.<sup>3</sup>

Now pipette into each cuvette 8.0 ml. of biuret reagent; mix, and read after 30 minutes as above.

Kjeldahl nitrogen determinations can be carried out on the remaining diluted serum. The total nitrogen content of 100 ml. of serum, corrected for non-protein nitrogen from a separate determination on the original serum, gives protein nitrogen which, multiplied by 6.25, is taken as the standard protein concentration.

Plot the transmission or density values observed against 0.15, 0.30, 0.45, 0.60, 0.80, 1.0, 1.2, and 1.4 times the protein concentration of the standard serum.

### Results

The calibration curves we have obtained using the reagent and the method described above obey satisfactorily the laws of Lambert and Beer.

<sup>3</sup> For work with 3.0 ml. samples use 0, 0.24, 0.45, 0.66, 0.9, 1.2, 1.5, 1.8, and 2.1 ml. of diluted serum, made to 3.0 ml. with saline. Plot the transmission values against 0, 0.16, 0.3, 0.44, 0.6, 0.8, 1.0, 1.2, and 1.4 times the protein concentration of the standard serum.

A slight loss of blue color must occur in the reaction because a proportion of the copper enters the protein "biuret" complex, but the effect on the extinction coefficient is scarcely apparent even at serum protein concentrations around 10 per cent. In Fig. 7 a typical calibration curve is shown, plotted on semilogarithmic paper. With the Evelyn colorimeter our  $K$  value  $((2 - \log G)/\text{concentration})$  has averaged about 580 with a range in any one curve of  $\pm 10$ .

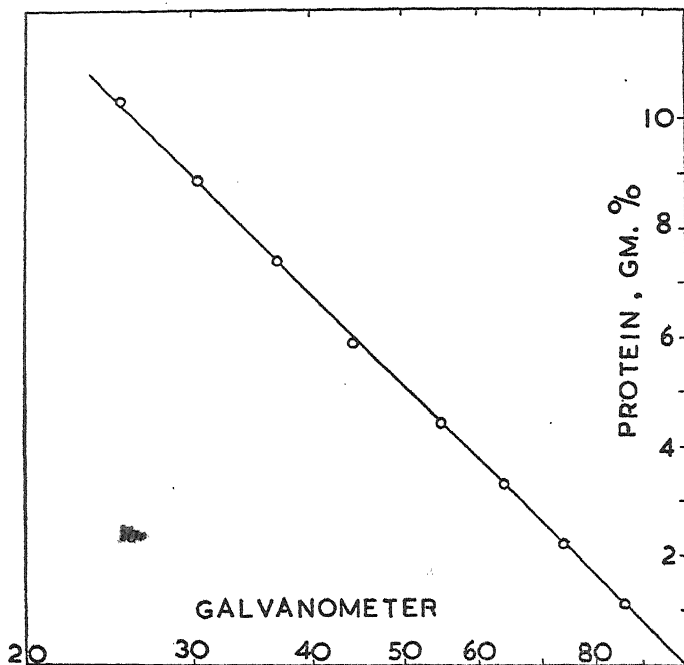


FIG. 7. Calibration curve for protein estimations with the biuret reagent, plotted on semilogarithmic graph paper.

Total protein and albumin analyses made with the modified biuret reagent have been compared with values calculated from Kjeldahl nitrogen determinations on the same sera; the results are shown in Table II. It can be seen that the biuret and Kjeldahl results can be expected to agree within about 0.1 gm. per cent of protein. The observed differences are statistically insignificant.

#### DISCUSSION

In our experience, the use of tartrate-stabilized biuret reagents offers a highly satisfactory method for estimating the proteins of serum or plasma.

The values obtained depend ultimately upon a Kjeldahl nitrogen determination, hence upon an ability to perform such an analysis accurately, and with the assumption of the factor 6.25. At present there appears to be no practical method of standardization superior to nitrogen analysis.

TABLE II  
*Results of Comparison of Total Protein and Albumin Analyses by Biuret and Kjeldahl Methods*

The values are measured in gm. per cent.

Serum		Diagnosis	Total protein		Albumin	
			Kjeldahl	Biuret	Kjeldahl	Biuret
Human	A. G.	Normal	7.49	7.35		
	C. B.	"	7.01	7.05		
	B. B.	"	7.04	7.05		
	J. D.	"	6.70	6.60		
	Pooled	"	7.45	7.35		
	H. G.	"	7.62	7.80		
	F.	Pyelonephritis	4.50	4.50	1.40	1.40
	R.	Pneumonia	7.12	6.95	3.79	3.81
	G.	Rheumatoid arthritis	6.80	6.70		
	C.	Cirrhosis	6.97	7.05		
	L.	Obstructive jaundice (serum bilirubin, 25 mg. %)	5.81	5.82		
	M.	Obstructive jaundice	5.91	6.07		
	E.	Lipoid nephrosis	3.85	3.65		
Dog No.	T.	Lupus erythematosus			3.25	3.20
	11	Normal	5.67	5.70		
	15	"	5.84	5.85		
	17	"	6.60	6.60		
	10	CCl <sub>4</sub> liver injury	6.08	5.95	3.35	3.24
	12	" " "	7.20	7.15		
	16	" " "	6.45	6.40		
	17	" " "			2.72	2.88
	48	Uremia (non-protein N = 200 mg. %)	5.06	5.15		

The procedure described for total protein determinations involves re-dissolving the globulins precipitated by sodium sulfate or other agents. No difficulty has been experienced with this technique, nor has the presence of sulfate any effect upon the rate or degree of color development. Sodium sulfite and methanol (at a final concentration of 2.1 per cent) may cause a very slight increase in color intensity, but for practical purposes the effect is negligible.

It is pertinent to comment briefly on a few special points.

*Interfering Factors*—There are practically no substances other than protein in biological fluids which give the biuret reaction, certainly none that cause significant interference. The pigment, bilirubin, absorbs light very weakly at 540 m $\mu$ . We confirm Kingsley's (6) observation that serum levels as high as 25 mg. per cent introduce (at the dilution employed) an error not greater than 0.1 gm. per cent protein. Although ammonium ion is a disturbing factor in the biuret reaction, the amount present when a mixture of ammonium and potassium oxalates is used as anticoagulant will not cause any significant error. With the method described, ammonium sulfate cannot be used as a globulin precipitant, but its interference can be minimized (a) by analysis of the separated precipitate or (b) by using a reagent containing 8 to 10 per cent alkali.

Careful separation of the serum or plasma should avoid the disturbing effect of hemolysis, and, if the patient is in the fasting state, the specimen will almost always be clear. There are certain diseases and situations, however, which give rise to lipemic sera and it is then necessary to vary the technique somewhat if the results are to have any significance. Such sera yield a somewhat cloudy reaction mixture and clearing must be effected before readings can be made. For this purpose we have used Kingsley's (6) method of adding 3 ml. of ether. The phases are mixed by flicking the tube for a definite period, say 10 or 20 seconds; the mixture is then centrifuged in a capped tube and the aqueous phase transferred to a new cuvette for reading at 30 minutes. This technique introduces a dilution error which in our hands has amounted to about 3 per cent. It is necessary for each worker to establish this correction, not only by measuring the change in volume, but by noting the optical density of a clear reaction mixture before and after treatment with ether. The use of petroleum ether (ligroin, b.p. 70–90°) will avoid the dilution error, though occasionally the two phases may not separate quite so readily.

*Age of Serum*—Wokes and Still (18) have reported that the intensity of the biuret color reaction increases rather strikingly as plasma ages. Our own experience is not in accord with their findings. If serum, or oxalated plasma, has been removed within a reasonable time ( $\frac{1}{2}$  to 2 hours) and stored under conditions in which it remains clear, no significant change has been observed in the protein concentration determined by the biuret reaction at  $\frac{1}{2}$ , 2, 4, 24, or 168 hours.

*Biuret Reaction with Different Proteins*—Although the exact nature of the "biuret" complex is still unknown, it is apparent that the accurate determination of serum protein fractions must presume a constant, average number of groupings which combine to give the reaction. Evidence supports the view that in this respect the biuret reaction is a more reliable quantitative method for protein estimation than one depending on the



content of some particular amino acid. Autenrieth (1) and Robinson and Hogden (5) regarded the color development by serum albumin and globulins as virtually identical. Fine (3), Kingsley (12), and Wokes and Still (18) have reported apparent differences in behavior of these two fractions in the biuret reaction.

We have determined the  $K$  values  $((2 - \log G)/C)$ , which are comparable to extinction coefficients, for the proteins listed in Table III. The protein concentration ( $C$ ) in each case has been calculated from a Kjeldahl nitrogen determination, corrected for non-protein nitrogen, and with the factor 6.25. The  $K$  values differ from the 580 previously mentioned because the reagent used for this experiment was of slightly different composition. It will be noted that, among the proteins tested, all the albumins and

TABLE III  
*K* Values for Different Proteins, Comparable to Extinction Coefficients

Nature of protein	$K = \frac{2 - \log G}{C}$
Human serum albumin	520
"    "    globulins	514
"    "    "abnormal" serum globulin (pptd. with 13.5% $\text{Na}_2\text{SO}_4$ )	515
"    "    serum (pooled)	520
Dog    "    "	517
Egg albumin	513
Casein	458
Zein	479
Gelatin (animal)	386

globulins show essentially identical values. The results with casein and zein differ to some extent, while gelatin shows a rather striking difference, one which was apparent visibly as a bluer tint.

The authors gratefully acknowledge the valued suggestions made by Dr. Andrew Hunter during the preparation of this report.

#### SUMMARY

Among the methods of preparing stable, "one piece" biuret reagents, the use of sodium potassium tartrate appears most promising.

The tartrate-stabilized reagent devised by Weichselbaum has been investigated in some detail and has been modified to contain less copper, more alkali, and less or no potassium iodide.

A simple procedure for the determination of serum total protein, albumin, and globulins is described.

The biuret reaction is approved as a simple, rapid, yet highly satisfactory and accurate method for the determination of the protein fractions in serum or plasma.

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# THE HYDROLYSIS OF N-BENZOYL-DL-TYROSYLGLYCINAMIDE, N-ACETYL-L-TYROSYLGLYCINAMIDE, AND N-ACETYL-DL-TYROSYLGLYCINAMIDE BY CHYMOTRYPSIN

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(Received for publication, August 16, 1948)

Contrary to the report of Bergmann and Fruton (1) the L peptide present in aqueous solutions of N-benzoyl-DL-tyrosylglycinamide, prepared by two independent methods, is rapidly hydrolyzed by crystalline chymotrypsin. At 40° and pH 7.8 in a 0.00344 M solution<sup>1</sup> of the DL mixture the L component is hydrolyzed as rapidly as in a 0.00172 M solution of the L peptide (Table I). Because of the relative insolubility of N-benzoyl-DL-tyrosylglycinamide, it was not possible to study the reaction at higher substrate concentrations, and attention was directed to the much more soluble N-acetyl-DL-tyrosylglycinamide.

The cryoscopic properties of N-acetyl-DL-tyrosylglycinamide in aqueous solution were determined over a concentration range varying from near saturation to approximately one-third of that value, and no indication was obtained suggestive of extensive interaction between the D and L peptides. Thus it can be concluded that in aqueous solutions of the DL peptide one is confronted with a simple mixture of the D and L peptides.

The chymotrypsin-catalyzed hydrolysis of the L peptide present in aqueous solutions of N-acetyl-DL-tyrosylglycinamide was studied, and at pH 7.8 and either 25° or 40° the rate of hydrolysis of the L component present in a 0.10 M solution of the DL peptide was found to be approximately one-half of the rate observed with a 0.050 M solution of the L peptide. At 40° in a solution 0.00172 M with respect to the L component the rates of hydrolysis were found to be identical, within experimental error, for solutions of the L and DL peptides. These data, summarized in Table II, clearly indicate that inhibition of hydrolysis of the L peptide by the D peptide is a function of enzyme-substrate concentrations and that the process is one involving competitive interaction between the D and L peptides and the enzyme. Thus under conditions of low substrate and inhibitor concentration the enzyme is unsaturated in the Michaelis sense and sufficient

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† Contribution No. 1236.

<sup>1</sup> A molar solution is defined for the purposes of this investigation as one containing 1 gm. formula weight per liter of solution.

reactive sites are available to permit optimum or near optimum hydrolysis of the L peptide, the presence of an equimolar quantity of the D peptide being without demonstrable effect. In solutions of high substrate and inhibitor concentration the enzyme can be considered to be operating at or near the limiting rate, and under these conditions interaction between the enzyme and the D peptide can cause substantial inhibition of the rate of hydrolysis of the L peptide. A more precise definition of the above system must await the accumulation of additional data.

Further study of the system involving N-benzoyl-DL-tyrosylglycinamide does not appear attractive, since it is clear that the limited solubility of this peptide and the relatively rapid rate of hydrolysis of the L component by chymotrypsin would prevent the attainment of conditions requisite for

TABLE I

*Hydrolysis of N-Benzoyltyrosylglycinamide at 40° and pH 7.8*

The DL substrate = 0.00344 M; the L substrate = 0.00172 M.

<i>E<sub>0</sub></i> , mg. protein N per ml.	Time of reaction	<i>S<sub>0</sub> - S</i>		First order rate constant, <i>k</i>		Proteolytic coefficient, <i>C</i>	
		DL	L	DL	L	DL	L
	<i>min.</i>	<i>mm per ml. × 10<sup>3</sup></i>	<i>mm per ml. × 10<sup>3</sup></i>				
0.0154*	10	0.66	0.70	0.049	0.052	3.2	3.4
	20	1.18	1.22	0.058	0.062	3.7	4.0
0.0154†	10	0.71	0.65	0.053	0.047	3.5	3.1
	20	1.24	1.15	0.063	0.055	4.1	3.6
	30	1.60	1.38				

\* Crystalline chymotrypsin preparation from Armour.

† Crystalline chymotrypsin preparation from Lehn and Fink.

the demonstration of inhibition by the D antipode. Practically there can be no objection to the use of N-benzoyl-DL-tyrosylglycinamide, which is much more readily available than its L component, as a test substrate for chymotrypsin-like activity.

In the hydrolysis experiments it was observed that a plot of the log of the substrate concentration *versus* time was generally linear, indicating that the reaction was approximately first order with respect to substrate. Rate constants and so called proteolytic coefficients (2) calculated by the commonly used first order rate expression are given in Tables I and II. Comparison of the proteolytic coefficients, which should be considered as approximate values because of possible complications arising from the comparatively slow hydrolysis of the terminal amide bond (3), shows that under comparable conditions N-benzoyl-L-tyrosylglycinamide is hydrolyzed by chymotrypsin much more rapidly than is the N-acetyl-L-peptide amide.

The kinetics of the hydrolysis of N-acetyl-L-tyrosylglycinamide by chymotrypsin at 25° and pH 7.8 were investigated and, assuming that hydrolytic cleavage of the terminal amide bond was so slow as to be ignored, it was found as in the chymotrypsin-catalyzed hydrolysis of N-benzoyl-L-tyrosinamide and ester (4) that the reaction was apparently first order at all initial substrate concentrations but that the first order rate constant ( $k$ ) increased with decreasing initial substrate concentrations. Two possible explanations for this behavior may be advanced. If inhibition by the

TABLE II  
*Hydrolysis of N-Acetyltyrosylglycinamide pH 7.8*

Temperature	$E_0^*$	$S_0^\dagger$	Time of reaction	$S_0 - S$ , mm per ml. $\times 10^3$		First order rate constant, $k$		Proteolytic coefficient, $C$	
				DL	L	DL	L	DL	L
°C.			min.						
40	0.0154	0.00172	30	0.29	0.36	0.0061	0.0079	0.39	0.51
			60	0.60	0.64	0.0071	0.0077	0.46	0.50
40	0.031	0.00172	20	0.45	0.42	0.015	0.014	0.45	0.45
			40	0.63	0.70	0.011	0.013	0.37	0.42
			60	0.90	0.85	0.012	0.011	0.40	0.36
40	0.060	0.05	20	7.4	12.8	0.0079	0.015	0.13	0.24
			40	13.5	22.0	0.0077	0.015	0.13	0.24
25	0.075	0.05	20	3.9	7.5	0.0041	0.0081	0.054	0.11
			40	8.1	14.2	0.0044	0.0083	0.058	0.11
			60		20.0		0.0035		0.11
25	0.30	0.05	80	13.7		0.0040		0.054	
			10	7.6	14.1	0.0165	0.0329	0.055	0.11
			20	12.6	24.8	0.0146	0.0342	0.049	0.11
			30		32.2		0.0342		0.11
			40	24.4		0.0167		0.056	

\* Mg. of protein N per ml.; crystalline chymotrypsin preparation from Lehn and Fink.

† Concentration of L form of substrate.

hydrolysis products occurs, the course of the reaction may be described by the expression

$$kEt = 2.3 \left[ K_m + \frac{1}{n} (S_0 + I) \right] \log \frac{S_0}{S} - \left( \frac{1-n}{n} \right) (S_0 - S)$$

where  $K_m$  = the Michaelis constant,  $n$  = the ratio of the inhibition constant to  $K_m$ ,  $I$  = the inhibitor concentration,  $S_0$  = the initial substrate concentration,  $S$  = the substrate concentration at time  $t$ , and  $E$  = the enzyme concentration. In the absence of inhibition by the hydrolysis products the rate law may be expressed by the integrated Michaelis-Menten equation (4)

$$kEt = 2.3K_m \log \frac{S_0}{S} + (S_0 - S)$$

The available data appear to fit either equation equally well and in the absence of data relative to possible inhibition by the hydrolysis products the simpler integrated Michaelis-Menten equation has been provisionally adopted for comparative purposes. In Table III are tabulated the experimental values of  $(S_0 - S)$  and those calculated from the integrated Michaelis-Menten equation with the best experimental values of  $K_m = 0.03$

TABLE III

*Comparison of Experimental and Calculated Results*

Temperature 25°,  $K_m = 0.03$  M,  $k = 0.0089$  mm per minute per mg. of enzyme nitrogen,  $E_0 = 0.15$  mg. of protein N per ml.

$S_0$	Time of reaction	Per cent hydrolysis	$S_0 - S$ (observed)	$S_0 - S$ (calculated)
mm per ml.	min.		mm per ml. $\times 10^3$	mm per ml. $\times 10^3$
0.05	10	14.9	7.4	8.1
	20	27.6	13.8	15.7
	40	48.6	24.3	27.3
0.03	10	16.9	5.1	6.3
	20	37.1	11.1	11.6
	40	65.2	19.6	20.0
0.02	10	26.1	5.2	4.9
	20	45.2	9.0	8.9
	30	59.4	11.9	12.4
0.0083	10	31.1	2.6	2.5
	20	53.2	4.4	4.3
	30	68.7	5.7	5.7
0.0050	10	31.0	1.6	1.6
	20	53.3	2.7	2.7
	30	70.6	3.5	3.5

M and  $k = 0.0089$  mm per minute per mg. of enzyme nitrogen. The agreement between experimental and calculated values of  $(S_0 - S)$  is within experimental error.

In view of the magnitude of  $K_m$  it appears likely that the extent of inhibition of hydrolysis of N-acetyl-L-tyrosylglycinamide by an equimolar quantity of the D antipode observed in solutions 0.10 M with respect to the DL peptide is at or near the limiting value.

## EXPERIMENTAL

*N-Benzoyl-DL-tyrosylglycinamide*—According to the procedure of Bergmann and Fruton (1), *p*-hydroxybenzaldehyde was condensed with hippuric acid, the azlactone allowed to react with glycine ester, and the dehydro-

peptide ester hydrogenated and ammoniated to give N-benzoyl-DL-tyrosylglycinamide trihydrate after recrystallization from aqueous ethanol.

*Analysis*— $C_{18}H_{19}O_4N_3 \cdot 3H_2O$ . Calculated. C 54.7, H 6.4, N 10.6  
Found. " 54.8, " 6.4, " 10.5

L-Tyrosine was converted into O,N-diacetyl-DL-tyrosine (5), the acetyl groups removed, and the DL-amino acid benzoylated with benzoyl chloride and sodium hydroxide to give O,N-dibenzoyl-DL-tyrosine, m.p. 215–217° (corrected) after recrystallization from aqueous ethanol.

*Analysis*— $C_{23}H_{19}O_5N$ . Calculated, N 3.6; found, N 3.7

A solution of 10 gm. of O,N-dibenzoyl-DL-tyrosine in 200 ml. of absolute ethanol was saturated with dry hydrogen chloride at 0°, the solution refluxed for 10 hours, the reaction mixture evaporated *in vacuo* to a thick syrup, the latter triturated with aqueous sodium bicarbonate, and the solid that formed recrystallized from aqueous ethanol to give 4.5 gm. of N-benzoyl-DL-tyrosine ethyl ester, m.p. 123–124° (corrected).

*Analysis*— $C_{18}H_{19}O_4N$ . Calculated, N 4.5; found, N 4.3

A solution of 8 gm. of N-benzoyl-DL-tyrosine ethyl ester in 20 ml. of absolute ethanol was slowly added to a refluxing solution of 40 ml. of 85 per cent hydrazine hydrate in 10 ml. of absolute ethanol, the reaction mixture refluxed for an additional 4 hours, chilled, and the recovered hydrazide washed with warm ethanol. Yield, 6.4 gm. of a product melting at 230–231° (corrected).

*Analysis*— $C_{16}H_{17}O_3N_3$ . Calculated. C 64.2, H 5.7, N 14.0  
Found. " 64.4, " 5.5, " 13.9

A solution containing 2.5 gm. of N-benzoyl-DL-tyrosinhydrazide, 5 ml. of concentrated hydrochloric acid, and 2.5 ml. of glacial acetic acid in 50 ml. of water was chilled to –10°, 10 ml. of a 7.5 per cent solution of sodium nitrite added with vigorous stirring, and the azide recovered, washed with cold water, dissolved in ethyl acetate, washed with aqueous sodium bicarbonate and water, and added to an ethereal solution of glycine ethyl ester prepared from 5 gm. of the hydrochloride. The reaction mixture was allowed to stand at 25° for 48 hours, then extracted with dilute hydrochloric acid and with water, the solvents removed, and the syrup triturated with a small amount of water. Recrystallization of the product from aqueous ethanol gave 2.0 gm. of N-benzoyl-DL-tyrosylglycine ethyl ester, m.p. 156–158° (corrected).

*Analysis*— $C_{20}H_{22}O_5N_2$ . Calculated. C 64.9, H 6.0, N 7.6  
Found. " 64.7, " 6.3, " 7.8

Ammonolysis of the above ester, in methanol solution, gave 1.8 gm. of N-benzoyl-DL-tyrosylglycinamide after recrystallization from aqueous ethanol.

*Analysis*— $C_{13}H_{19}O_4N_3 \cdot 3H_2O$ . Calculated. C 54.7, H 6.4, N 10.6  
Found. " 54.9, " 6.6, " 10.5

*N-Benzoyl-L-tyrosylglycinamide*—This acyldipeptide amide was prepared according to the directions of Bergmann and Fruton (1). The product was obtained as fine needles, m.p. 218–219° (decomposition).

*N-Acetyl-DL-tyrosylglycinamide*—A solution of 25 gm. of N-acetyl-DL-tyrosine ethyl ester (6) in 60 ml. of absolute ethanol was slowly added to a refluxing solution of 12.5 gm. of 85 per cent hydrazine hydrate in 20 ml. of absolute ethanol, and the reaction mixture refluxed for an additional 2 hours, chilled, the solid recovered, washed with ethanol, and dried to give 21 gm. of N-acetyl-DL-tyrosinhydrazide, m.p. 227–227.5° (corrected).

*Analysis*— $C_{11}H_{15}O_3N_3$ . Calculated. C 55.6, H 6.4, N 17.7  
Found. " 55.5, " 6.1, " 17.7

A solution of 1.5 gm. of sodium nitrite in 10 ml. of water was added to a well stirred solution, maintained at  $-5^\circ$ , containing 4.0 gm. of N-acetyl-DL-tyrosinhydrazide, 10 ml. of concentrated hydrochloric acid, and 5 ml. of glacial acetic acid in 50 ml. of water. The azide was recovered, washed with cold water, taken up in ethyl acetate, and the latter solution washed with aqueous sodium bicarbonate and water, and added to an ethereal solution of glycine ethyl ester prepared from 10 gm. of the hydrochloride. The reaction mixture was washed with dilute hydrochloric acid and with water, and the solvents removed from the dried non-aqueous phase to give 3.5 gm. of N-acetyl-DL-tyrosylglycine ethyl ester, m.p. 135–136° (corrected), after recrystallization from aqueous ethanol.

*Analysis*— $C_{13}H_{20}O_5N_2$ . Calculated. C 58.5, H 6.6, N 9.1  
Found. " 58.4, " 6.5, " 9.0

Ammonolysis of 2.0 gm. of the above ester, in methanol solution, gave 1.2 gm. of N-acetyl-DL-tyrosylglycinamide, m.p. 205–206.5° (corrected) after recrystallization from aqueous ethanol.

*Analysis*— $C_{13}H_{17}O_4N_3$ . Calculated. C 55.9, H 6.1, N 15.1  
Found. " 56.1, " 6.2, " 15.3

*N-Acetyl-L-tyrosylglycinamide*—A solution of 37 gm. of N-acetyl-L-tyrosine (5) in 500 ml. of absolute ethanol was saturated with dry hydrogen chloride at  $0^\circ$ , the solution refluxed for 4 hours, the solution concentrated *in vacuo* to a thick syrup, the pH adjusted to 7.5 with aqueous sodium



carbonate, and the crude product recrystallized from aqueous ethanol to give 25 gm. of N-acetyl-L-tyrosine ethyl ester, m.p. 96–97° (corrected).

*Analysis*— $C_{13}H_{17}O_4N$ . Calculated. C 62.1, H 6.8, N 5.6  
Found. " 61.8, " 6.9, " 5.3

According to the procedure used for the DL peptide, 22 gm. of the above ester gave 17 gm. of N-acetyl-L-tyrosinhydrazide, m.p. 235.5–236° (corrected).

*Analysis*— $C_{11}H_{15}O_3N_2$ . Calculated. C 55.6, H 6.4, N 17.7  
Found. " 55.4, " 6.5, " 17.8

N-Acetyl-L-tyrosinhydrazide was converted into N-acetyl-L-tyrosylglycine ethyl ester, m.p. 133–135° (corrected) in a manner similar to that described for the preparation of the corresponding DL peptide. The yields varied between 55 and 75 per cent of the theoretical.

TABLE IV  
*Molecular Weight of N-Acetyl-DL-Tyrosylglycinamide in Aqueous Solution*

Peptide in 1000 gm. water	Freezing point depression	Mol. wt.
gm.	°C.	
21.40	0.137	290
21.00	0.137	284
10.50	0.070	278
10.30	0.068	281
6.17	0.038	301

*Analysis*— $C_{13}H_{20}O_5N_2$ . Calculated. C 58.5, H 6.6, N 9.1  
Found. " 58.6, " 6.9, " 9.4

Ammonolysis of the above ester gave 78 per cent of N-acetyl-L-tyrosylglycinamide, m.p. 225–226° (corrected).

*Analysis*— $C_{13}H_{17}O_4N_3$ . Calculated. C 55.9, H 6.2, N 15.1  
Found. " 55.9, " 5.9, " 15.0  
 $[\alpha]_D^{25} = +35.0^\circ$  (4% in 50% aqueous acetone)

*Cryoscopic Measurements*—The technique described by Beckmann (7) was used for the determination of the freezing points of aqueous solutions of N-acetyl-DL-tyrosylglycinamide. The molecular weights given in Table IV are to be compared with the theoretical value of 279, assuming ideal solution behavior.

*Enzymatic Studies*—Preparations of crystalline chymotrypsin obtained from Lehn and Fink or Armour were used in these studies and were found to give comparable results. The reaction mixtures were 0.015 M in phosphate, adjusted to pH 7.8. The extent of hydrolysis was determined by a formol

titration to pH 8.1 with a Beckman model G pH meter for indication of the end-point. In general 0.5 ml. aliquots of the 0.05 M substrate solutions were titrated with 0.01 M sodium hydroxide after the addition of 0.5 ml. of 35 per cent aqueous formaldehyde which had been adjusted to pH 8.0 by the addition of basic magnesium carbonate. With the 0.0017 M substrate solutions 2.0 ml. aliquots were titrated.

#### SUMMARY

The inhibition of the chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tyrosylglycinamide by equimolar quantities of its D antipode has been observed to be a function of the relative concentration of the DL mixture and enzyme. The general features of the above system have been described, and an explanation has been given relative to the lack of antipodal inhibition obtaining in the case of N-benzoyl-DL-tyrosylglycinamide.

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# OXIDATION-COUPLED INCORPORATION OF INORGANIC RADIOPHOSPHATE INTO PHOSPHOLIPIDE AND NUCLEIC ACID IN A CELL-FREE SYSTEM\*

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(Received for publication, August 16, 1948)

There is a considerable body of evidence concerning the participation of acid-soluble phosphate esters, such as the adenylic acid system, phosphocreatine, etc., in the esterification of inorganic phosphate coupled to oxidations of the Krebs tricarboxylic acid cycle in cell-free systems. However, no comparable studies *in vitro* have been reported on the participation of acid-insoluble phosphate esters such as the phospholipides and pentose and desoxypentose nucleic acids in the process of aerobic phosphorylation, either as primary participants or as secondary phosphate acceptors from the adenylic acid system. There are some experimental indications that the phosphorylation of phospholipides depends on respiration as a source of energy (1). Spiegelman and Kamen (2) have called attention to a possible rôle of nucleoprotein as an energy transmitter between reactions of catabolism and the synthesis of protein.

The investigation reported in this paper represents an exploration into the possibility of studying the incorporation of inorganic phosphate into phospholipide and nucleic acid molecules under the same conditions that have been used to demonstrate the phosphorylation of such acceptors as creatine, glucose, etc., in cell-free systems coupled to oxidations of the Krebs tricarboxylic acid cycle.

The experimental material chosen for this study was a washed, cell-free suspension of the particulate material of rat liver, which previous work has demonstrated to catalyze all the oxidations of the Krebs cycle, fatty acid oxidation, and coupled esterification of phosphate (3). These preparations are composed largely of mitochondria and nuclei. Other work has shown that the mitochondria of rat liver, as isolated by differential centrifugation (4), are the major site of these highly complex respiratory sys-

\* Aided by grants from the American Cancer Society (recommended by the Committee on Growth of the National Research Council) and Mr. Ben May, Mobile, Alabama. The work reported here was taken from a thesis submitted by Morris Friedkin to the Division of Biological Sciences of the University of Chicago in partial fulfillment of the requirements for the degree of Doctor of Philosophy, September, 1948.

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tems (5). The mitochondria contain phospholipides and pentose nucleic acid as integral structural components (6, 7). In view of the highly organized nature of the mitochondria, it appeared possible that the phospholipides and nucleic acid of these structures are in a strategic morphological position to serve as phosphate acceptors during active oxidative phosphorylation in these particles. Therefore a study was initiated of the incorporation of inorganic phosphate labeled with  $P^{32}$  into the phospholipides and nucleic acids of washed particulate suspensions of rat liver (properly supplemented with  $Mg^{++}$ , adenine nucleotide) which were actively carrying on oxidations of the Krebs tricarboxylic acid cycle. Phospholipide and nucleic acid fractions of the particulate material were then separated and analyzed to determine  $P^{32}$  incorporation. Since the amounts of phospholipide and nucleic acid present in such preparations are insufficient for actual isolation and rigorous purification to constant specific activity, it was necessary to adapt the analytical separations devised by Schneider (8) and Schmidt and Thannhauser (9) to the requirements of the isotope technique. Suitable control procedures established the validity of the measurements.

In this paper are presented complete data on one experiment of a series performed under nearly the same conditions and showing quantitatively similar results.

#### EXPERIMENTAL

*Procedures*—Early shipments of radioactive phosphorus were obtained in the form of  $Na_2HP^{32}O_4$  through the United States Atomic Energy Commission. Later shipments, received as  $H_3P^{32}O_4$ , pH 4, appeared to contain acid-labile esterified or anhydride  $P^{32}$  and were therefore treated with 1 N HCl at  $100^\circ$  for 10 minutes before neutralization. Radioactivity measurements were made under conditions of constant geometry on aqueous solutions (1.0 ml. in aluminum cups, round, flat bottom; diameter  $\frac{3}{4}$  inch, depth  $\frac{1}{4}$  inch) with a standard Geiger-Müller tube and a Cyclotron Specialties recorder (scale-of-thirty-two). This technique of measuring radioactivity in solution was found to yield a directly linear relationship between concentration of  $P^{32}$  and counts under our conditions.

Manometric measurements were made in air at  $30^\circ$  with Warburg vessels equipped with alkali and filter paper roll in the center well. All components of the reaction mixture were present in the main compartment of Warburg vessels, which were placed in cracked ice. Ice-cold enzyme was added last. A 4 minute temperature equilibration period preceded manometric measurements.

Inorganic and total phosphorus was determined by the Fiske and Subbarow method as modified by Gomori (10).

*Preparation of Enzyme*—The enzyme preparation used consisted of a water suspension of the washed particulate matter of rat liver, prepared by a modification of a method described by Lehninger and Kennedy (3). 5 gm. of chilled rat liver, freshly removed from the exsanguinated animal, were homogenized with 8.0 ml. of ice-cold modified Krebs-Ringer buffer, pH 7.8 (11). The homogenate was strained through gauze and diluted with an equal volume of cold buffer. The diluted mixture was centrifuged at  $2200 \times g$  in an angle head centrifuge in the cold for 3 minutes. The supernatant was discarded. Fresh cold buffer was added to the residue to equal the original volume of the diluted homogenate. The residue was resuspended by shaking and again centrifuged in the cold (3 minutes,  $2200 \times g$ ). The supernatant was decanted and the residue again taken up in the same volume of buffer and centrifuged. After the supernatant from

TABLE I

*Distribution of Acid-Insoluble P in Typical Enzyme Preparation*

The values represent micrograms of P found in each P fraction of the amount of enzyme suspension used in a single Warburg vessel (originally derived from 0.33 mg. of whole rat liver).

Fraction	P content
Phospholipide. ....	114
Total nucleic acids. ....	51.5
Desoxypentose nucleic acid. ....	25.0
Phosphoprotein residue. ....	10.5

the last washing was decanted, the residue was taken up in cold redistilled water to 12 ml. The water suspension thus prepared was equivalent, for the amount used in each Warburg flask (0.8 ml.), to 0.33 gm. of whole fresh rat liver. This preparation consists largely of a mixture of mitochondria and nuclei and contains phospholipides and both pentose and desoxypentose nucleic acids bound in the particulate material. The distribution of acid-insoluble phosphate esters in a typical enzyme preparation is given in Table I.

*Conditions for Phosphorylation*—With components of the enzyme reaction mixtures as indicated in Table II, four different experimental conditions were compared for the degree of incorporation of  $P^{32}$  into the various phosphate ester fractions.

*Zero Time Control*—Trichloroacetic acid was added prior to addition of enzyme to the reaction mixture (malate omitted).

*Inactivated Enzyme*—3 ml. of freshly prepared enzyme were shaken in a Warburg flask at  $30^\circ$  for 3 hours. During this period 120 microliters of oxygen were consumed. Toward the end of the 3 hour period the oxygen

uptake had ceased completely. An aliquot of the inactivated enzyme was then added to the reaction mixture (malate omitted). This aged enzyme is completely inactive in catalyzing oxidative phosphorylation coupled to the Krebs cycle.

*System without Malate*—Fresh enzyme was added to the reaction mixture which contained no added substrate.

*Complete System*—Fresh enzyme was added to the complete reaction mixture containing malate as substrate, as indicated in Table II. Mano-

TABLE II

*Incorporation of Inorganic Phosphate Labeled with P<sup>32</sup> into Acid-Soluble Esterified Phosphate Fraction Coupled to Oxidation of Malate*

The main compartment of the Warburg vessels contained 0.8 ml. of enzyme preparation, 0.20 ml. of KCl (0.05 M),\* 0.20 ml. of MgSO<sub>4</sub> (0.005 M), 0.20 ml. of glycylglycine buffer, pH 7.3, (0.01 M), 0.20 ml. of ATP (0.00067 M), 0.20 ml. of malate (0.005 M), and 2,110,000 counts per minute of P<sup>32</sup> (28.6 microcuries) as inorganic phosphate in a total volume of 2.0 ml. Incubated in air at 30° for 4 minutes; taps closed and manometric measurements made for the following 19 minutes.

	Experimental conditions			
	Zero time	Inactivated enzyme	System without malate	Complete system
Oxygen uptake, <i>microliters</i>		-2	16	108
		-2	32	110
Inorganic P, $\gamma$	140	294	246	164
	144	288	252	160
Esterified " "	146	22	40	114
	142	16	34	114
P <sup>32</sup> incorporated into esterified P, %	0.12	0.44	2.46	21.0
	0.12	0.63	1.69	20.9
Specific activity of esterified P, <i>counts per min. per <math>\gamma</math> P</i>	28	443	1440	5080
	30	554	1050	4880

\* The figures in parentheses refer to final concentration of the component in the complete reaction medium.

metric measurements were then carried out according to the conditions outlined in Table II. After completion of the incubation the reactions were stopped by the addition of 2.5 ml. of 10 per cent trichloroacetic acid (TCA) and the flask contents subjected to analysis as outlined below.

*Method for Separation of Phosphate Fractions*—In view of the special precautions required for using radioactive phosphorus, several modifications of the methods described by Schneider (8, 12) and by Schmidt and Thannhauser (9) for separation of phospholipide and nucleic acid fractions were necessary to eliminate contamination by inorganic P<sup>32</sup>. The method for separation of the various phosphate fractions will therefore be described

in detail. All operations were carried out in a cold room at 2° whenever possible. Residues and precipitates were resuspended by means of magnetic stirring. The stirrer was a small elongated pellet consisting of an iron wire sealed in glass. The pellet was not removed during the centrifugation steps.

### *Steps of Partition*

1. To the enzyme reaction mixture were added 2.5 ml. of 10 per cent TCA at the completion of the incubation period. After 20 minutes at 0°, the contents of the Warburg flask were poured into a 15 ml. conical tube and centrifuged.

2. The Warburg flask was rinsed with two portions of 1.25 ml. of 10 per cent TCA. The residue from Step 1 was suspended in the rinse, mixed, and centrifuged.

3. The supernatants from Steps 1 and 2 were combined and diluted with water to 10.0 ml. This solution contained *acid-soluble P*.

4. The trichloroacetic acid precipitate was washed three times by resuspension in 2.5 ml. of TCA containing carrier inorganic phosphate (0.04 M  $\text{NaH}_2\text{PO}_4$  in 10 per cent TCA) and centrifugation. The washings were usually counted for radioactivity as a control measure to determine the extent of removal of contaminating inorganic  $\text{P}^{32}$  and then discarded.

5. The washed residue was taken up in 1.0 ml. of  $\text{H}_2\text{O}$ . 4 ml. of 95 per cent ethanol were then added. After extraction at room temperature the mixture was centrifuged.

6. The residue was again extracted with 5.0 ml. of 95 per cent ethanol at room temperature.

7. The residue was reextracted with two 5.0 ml. portions of 3:1 ethanol-ether for 3 minutes at 60°.

8. The supernatants from Steps 5, 6, and 7 were combined and the *phospholipide* contained in this solution was then subjected to further purification as described later.

9. Insoluble residue from Step 7 was extracted once more with 5.0 ml. of 3:1 ethanol-ether for 3 minutes at 60°. This extract was found to contain negligible radioactivity and was discarded.

Separations through Steps 1 to 9 were carried out in duplicate, each set of two tubes corresponding to a pair of duplicate flasks for each experimental condition as outlined before. Beginning with Step 10, one tube of each set was subjected to the Schmidt-Thannhauser partition and the other (beginning at Step 17) to the Schneider procedure.

10. The residue from Step 9 was taken up in 2.0 ml. of 1 M KOH and kept at 37° for 20 hours. The tube was then chilled in an ice bath; 0.4 ml. of 6 N HCl (cold) and 3.0 ml. of 5 per cent TCA (cold) were added,

and, after 10 minutes at 0°, the precipitated desoxypentose nucleoprotein was separated by centrifugation.

11. Insoluble residue from Step 10 was washed with 2.5 ml. of 5 per cent TCA.

12. The supernatants from Steps 10 and 11 were combined and diluted to 10.0 ml. with water. This solution contained *pentose nucleic acid and "phosphoprotein" P* fractions.

13. The residue from Step 11 was washed twice with 2.5 ml. of cold 5 per cent TCA. The supernatants were discarded after counting had shown no appreciable radioactivity.

14. The residue from Step 13 was extracted with 5.0 ml. of 5 per cent TCA for 15 minutes (100° water bath), cooled, and centrifuged.

15. The residue from Step 14 was washed with 2.5 ml. of cold 5 per cent TCA.

TABLE III  
Main Phosphate Fractions Isolated by Procedure

Step No.	Phosphorus fraction
3	Acid-soluble
8	Phospholipide
12	Pentose nucleic acid and phosphoprotein
16	Desoxypentose nucleic acid
19	Total nucleic acid
21	"Phosphoprotein" residue

16. The supernatants from Steps 14 and 15 were combined and diluted with water to 10.0 ml. This solution contained *desoxypentose nucleic acid P*.

17. Beginning of Schneider procedure on separate tube. The residue from Step 9 was extracted with 5.0 ml. of 5 per cent TCA for 15 minutes (water bath at 100°). The tube was then chilled and centrifuged.

18. Insoluble residue from Step 17 was washed with 2.5 ml. of cold 5 per cent TCA.

19. The supernatants from Steps 17 and 18 were combined and diluted with water to 10.0 ml. This solution contained *total nucleic acid P*.

20. The residue from Step 18 was extracted three times with 2.5 ml. portions of 5 per cent TCA for 15 minutes at 100°. Determination of radioactivity of these extracts is commented on later.

21. The residue from Step 20 was digested in sulfuric acid for subsequent total phosphate determination and counting. This fraction contained residual "*phosphoprotein*" *P*.

A résumé of the identity of the main fractions separated by this procedure,



in terms of the Schmidt-Thannhauser and Schneider methods, is given in Table III. These fractions were analyzed for total phosphorus and for radioactivity after appropriate purification, as outlined below.

*Further Purification of Main Fractions to Remove Contaminating Inorganic P<sup>32</sup>*

*Separation of Inorganic Phosphate from Acid-Soluble Esterified Phosphate*—Conditions for the separation of inorganic phosphate from esterified phosphate by precipitation with magnesia mixture must be well controlled to prevent coprecipitation of esterified phosphate. In some trial separations of inorganic phosphate from adenosine triphosphate (ATP) (1 micromole in 5.0 ml. of 5 per cent TCA), it was found that not more than 5 micromoles of inorganic phosphate should be present for good recovery of esterified P in the supernatant. With concentrations above this level a considerable loss of ATP occurs because of coprecipitation. Of several magnesia mixtures investigated, one containing MgSO<sub>4</sub> gave the best results. The following illustrates the technique of magnesia precipitation used.

All of the following operations were carried out at 0–2°. To 5.0 ml. of 5 per cent TCA extract (Step 3, acid-soluble P fraction) were added 0.05 ml. of 0.1 M phosphate (5 micromoles of inorganic phosphate), 0.2 ml. of 15 N NH<sub>3</sub>, and 1.0 ml. of magnesia mixture (0.1 M MgSO<sub>4</sub>–1 M NH<sub>4</sub>Cl). After standing overnight in a refrigerator, the tube was centrifuged for 15 minutes. The supernatant was carefully decanted into another conical tube containing 5 micromoles of inorganic phosphate and was acidified by addition of 0.1 ml. of 10 N H<sub>2</sub>SO<sub>4</sub>. After thorough mixing, 0.2 ml. of 15 N NH<sub>3</sub> was added and the tube allowed to stand in a refrigerator from 4 to 8 hours. The tube was then centrifuged for 15 minutes. Aliquots of the supernatant were taken for counts and for phosphate determinations and corresponded to acid-soluble esterified P, essentially free of contaminating inorganic P<sup>32</sup>.

Recovery of esterified phosphate after two magnesia precipitations with carrier inorganic phosphate as described was 60 to 70 per cent, depending on the amount of inorganic phosphate originally present. No method was found for precipitation of inorganic phosphate with magnesia mixture, which gives higher yields of esterified phosphate. In all tabulations of per cent incorporation of P<sup>32</sup> into acid-soluble esterified phosphate, the values given are minimal values, not corrected for loss of esterified phosphate due to coprecipitation with inorganic phosphate. It is possible to correct for the loss with the specific activity of the esterified phosphate known; however, there is no definite evidence that coprecipitated and supernatant esterified phosphate are completely homogeneous with respect to composition and

specific activity. The per cent loss of esterified phosphate following the above treatment in tubes representing the four different experimental conditions outlined above was consistent and made possible a qualitative evaluation which was correlated in all cases with specific activities of esterified P in each fraction.

*Purification of Phospholipide*—The phospholipide fraction as obtained directly from the fractionation procedure was grossly contaminated with inorganic  $P^{32}$  despite the repeated washes with trichloroacetic acid. Special precautions were therefore necessary for the removal of this contaminant. The procedure used for removal of inorganic  $P^{32}$  was based on a method used by Fishler *et al.* (13) in their studies on plasma phospholipide turnover.

The alcohol-ether extract from Step 8 was evaporated in the presence of 1.0 ml. of 1 M  $Na_2HPO_4$  under nitrogen *in vacuo* at 50° until 1.0 ml. of aqueous residue remained. The residue was extracted three times with 25 ml. portions of ethyl ether. The ether extracts were combined and reduced in volume with 1.0 ml. of 1 M  $Na_2HPO_4$ , again added as above. The residue which remained was extracted three times with 25 ml. portions of ethyl ether. The ether extracts were combined and diluted to 100 ml. 1 ml. was taken to dryness in an aluminum cup and counted. The "dry" counts were converted to "wet" counts. A "wet" count is the count for 1.0 ml. of aqueous solution of  $P^{32}$ . A "dry" count is the count of the same sample evaporated to dryness. For the particular geometry of the counter used (wet counts)/(dry counts) = 0.558. The phospholipide fraction purified in the manner outlined contains negligible amounts of contaminating  $P^{32}$ .

### Results

*Incorporation of  $P^{32}$  into Acid-Soluble Esters Coupled to Malate Oxidation*—Table II is a protocol describing the details of the manometric experiment, oxygen uptakes, the amounts of acid-soluble inorganic and esterified P, the amounts of  $P^{32}$  incorporated into the acid-soluble esterified P fraction in terms of per cent inorganic  $P^{32}$  originally added, and the specific activity of the esterified P.

Usually in experiments designed to demonstrate aerobic phosphorylation some phosphate acceptor such as glucose, creatine, etc., is added to a respiring system in order to cause a *net* accumulation of newly esterified P. The enzyme preparations used in this experiment do not contain the necessary transphosphorylases for reaction between ATP and such acceptors. In the absence of such acceptor systems newly formed ATP is rapidly hydrolyzed, owing to the action of phosphatases. However, in these experiments the rate of hydrolysis does not greatly exceed the rate of synthesis coupled to the oxidation of malate. As a result the level of esterified

phosphate is maintained in the presence of oxidation of malate, whereas in the absence of coupled oxidations the ATP originally added is quickly hydrolyzed. If these reactions are carried out in the presence of inorganic  $P^{32}$ , then the esterified P should show rapid incorporation of inorganic  $P^{32}$  coupled to oxidations. In Table II the results on the acid-soluble P exchanges illustrate this analysis. In the complete system oxidizing malate the level of esterified P is maintained near the level of the amount originally added as ATP, shown in the zero time experiment. This esterified P now contains 21 per cent of the inorganic  $P^{32}$  originally added, whereas the experiment at zero time shows only 0.12 per cent  $P^{32}$  incorporation. The latter figure indicates the completeness of separation of inorganic from organic phosphate by the methods used. The aged, inactivated enzyme preparation caused only a trace of incorporation of  $P^{32}$ , which may not be

TABLE IV  
*Incorporation of  $P^{32}$  into Phospholipides*

Phospholipide was extracted into ethyl ether and equilibrated with carrier disodium phosphate as described in the text.

	Experimental conditions			
	Zero time	Inactivated enzyme	System without malate	Complete system
Incorporation, %	0.003	0.007	0.072	0.132
	0.008	0.003	0.040	0.138
Specific activity, counts per	0.3	1.0	10.5	19.6
min. per $\gamma$ P	1.0	0.5	6.0	20.4

significant. In the system containing no added malate some 2.5 per cent of  $P^{32}$  was incorporated and a small degree of maintenance occurred. These changes are probably due to oxidation of traces of substrates present in the enzyme preparation.

These findings demonstrate that the complete system shows active aerobic phosphorylation coupled to the oxidation of a Krebs cycle intermediate. Furthermore, the data also provide control conditions (zero time, inactivated enzyme, and system without malate) which serve as a base-line for evaluating the significance of incorporations of  $P^{32}$  into phospholipide, nucleic acid, and "phosphoprotein" fractions outlined below.

*Incorporation of  $P^{32}$  into Phospholipide P*—In Table IV are shown data on the incorporation of  $P^{32}$  into the phospholipides contained in the enzyme preparation in the experiments outlined in Table II. It can be seen that incorporation of  $P^{32}$  into the phospholipide fraction occurred only in the presence of oxidation and to its greatest extent when malate was being oxidized. The finding that no incorporation of  $P^{32}$  occurred in the experi-

ment at zero time and in the inactivated enzyme provides an indication of the success of the method of separating traces of contaminating inorganic  $P^{32}$  from the phospholipid fraction. It will be noted that the specific activity of the phospholipides is much lower than the specific activity of the acid-soluble esterified P (Table II), indicating a much lower rate of turnover.

*Incorporation of  $P^{32}$  into Nucleic Acids*—Despite many extractions of the trichloroacetic acid residue to remove contaminating inorganic  $P^{32}$ , a considerable amount of inorganic  $P^{32}$  was apparently retained by the residue, as evidenced by the relatively high  $P^{32}$  content of the total nucleic acid fraction of the zero time control in which no true incorporation was expected (see Table V, Step 19).

TABLE V  
*Incorporation of  $P^{32}$  into Nucleic Acids*

	Per cent of $P^{32}$ originally added			
	Zero time	Inactivated enzyme	System without malate	Complete system
Total nucleic acid P (Step 19)	0.316	0.417	0.423	0.506
After magnesia treatment	0.007	0.039	0.086	0.121
Desoxypentose nucleic acid P (Step 16)	0.019	0.037	0.045	0.070
After magnesia treatment	Trace	Trace	Trace	Trace
Pentose nucleic acid and residue P (Step 12)	0.200	0.390	0.507	0.650
After magnesia treatment	0.002	0.027	0.098	0.166

In order to detect the activity of true esterified P in the nucleic acid fractions, counts were taken after treatment of the crude fractions with carrier inorganic phosphate and a single precipitation with magnesia mixture. The supernatants, essentially free of inorganic  $P^{32}$ , were then counted.

The values, shown in Table V, represent minimal incorporations due to some loss of esterified P during the magnesia treatment.

The desoxypentose nucleic acid (DNA) fraction, treated in the same way, showed no significant radioactivity. With incorporation into DNA almost nil, the radioactivity of the total nucleic acid fraction can be attributed to the pentose nucleic acids (PNA). However, the extraction of additional radioactivity upon further treatment of the residue with portions of hot TCA, shown in Table VI, indicates either that the first extraction did not completely remove total nucleic acids or that the "phosphoprotein" residue remaining is in part labile to hot TCA. Schneider (8) has found that a

single extraction is enough to remove all of the nucleic acid as measured by ribose determinations. In view of this fact it is possible that the phosphorus of the residue, which has a high specific activity, is not completely stable to hot TCA.

In the Schmidt-Thannhauser method, treatment with alkali converts PNA into mononucleotides and releases inorganic phosphate from phosphoprotein. After separation of inorganic phosphate the radioactivity of the esterified phosphate should represent PNA and provide a check on the activity of the total nucleic acid fraction. The incorporation of  $P^{32}$  into this fraction (Table V, Step 12 after magnesia treatment) is of the same order as that of the total nucleic acid by the Schneider procedure.

TABLE VI  
*Incorporation of  $P^{32}$  into Unidentified "Phosphoprotein" Residue*

	Zero time	Inactivated enzyme	System without malate	Complete system
Step 20, counts per min.				
Extract 1	83	115	695	1570
" 2	13	31	452	1080
" 3	0	6	263	577
Step 21, counts per min.	0	63	775	1750
Total .....	96	215	2185	4977
$P^{32}$ incorporated (Steps 20-21), %	0.005	0.010	0.104	0.236
Specific activity (Step 21), counts per min. per $\gamma$ P	0	4.8	73	146

It may therefore be concluded on the basis of these independent approaches that the radioactivities of the pentose nucleic acid fraction are probably valid measurements. It can be seen that the incorporation of  $P^{32}$  into the pentose nucleic acid is correlated with active oxidation, as is the incorporation into phospholipide.

The specific activity of the nucleic acid P was not determined in this experiment; however, in a comparable experiment the data showed that the rate of turnover for pentose nucleic acid P was quite low compared to that for the acid-soluble esters, but about 5 times the magnitude of the turnover of phospholipide P. Specific activities before magnesia treatment were as follows: (1) zero time control, total nucleic acid 3.9; (2) system without malate, total nucleic acid, 50.5; (3) complete system, total nucleic acid, 53.5; pentose nucleic acid, 93.0; desoxypentose nucleic acid, 11.0.

*Incorporation of  $P^{32}$  into Unidentified "Phosphoprotein" Residue*—After

the first hot TCA extraction of total nucleic acid (Step 17), three successive hot TCA extractions were carried out to determine the stability of the counts which remained in the residue. The results, shown in Table VI, indicate that, whereas few counts are incorporated into the "phosphoprotein" residue in the inactivated system, a significant incorporation has occurred in the system without malate and in the complete system, again correlated with turnover in the acid-soluble P fraction and with oxidation. The radioactivity extractable with hot TCA (Step 20) was not characterized as to the organic or inorganic nature of the phosphate. The activity of the final "phosphoprotein" residue not only exhibited a relationship to oxidation but also possessed the highest specific activity encountered in the acid-insoluble esters, although it was still small compared to the activity of the acid-soluble esters.

#### DISCUSSION

It must be clearly understood that the method of separation of phospholipides and nucleic acid fractions employed in this investigation cannot be regarded as a completely adequate substitute for the accepted ideal of isolating the desired component and purifying it to constant activity by recrystallization, etc. The amounts of the acid-insoluble esters available in the type of experiments described, even if they were performed on a much larger scale, are far too small for exhaustive purification. Furthermore, the difficulties in obtaining nucleic acids and phospholipides in a state approaching purity by acceptable physical and chemical criteria even on a preparative scale are well known. In view of the fact that the analytical procedures of Schneider (8) and Schmidt and Thannhauser (9), on which the separations were based, show excellent agreement in the analysis of rat liver (12), which is the source of the enzyme preparations used in this study, the results obtained by solubility separations may be regarded as semiquantitatively valid. In addition the very low radioactivities of fractions derived from the zero time and inactivated systems provide considerable assurance of the effectiveness and validity of the separations.

Two main facts emerge from the data. First, they show that incorporation of inorganic phosphate into phospholipide, pentose nucleic acid, and "phosphoprotein" can occur in a cell-free system which is so constituted as to show extensive aerobic phosphorylation of the adenylic acid system coupled to oxidations in the Krebs tricarboxylic acid cycle. The dependence of these phosphorylations on oxidation supports the findings and views of many investigators on the relationship between biosynthetic reactions and respiration as the primary source of energy.

Second, the relative rates of incorporation of the inorganic  $P^{32}$  into acid-soluble P, phospholipide P, and nucleic acid P fractions observed in the

cell-free system studied are roughly of the same relative order of magnitude as those observed in studies of intact animal tissues *in vivo*. Although it is difficult to compare such data quantitatively, consideration of the *in vivo* findings of Kalckar *et al.* (14) on ATP turnover, of Chaikoff (15) and Hahn and Tyrén (16) on phospholipid turnover, and of Hammarsten and Hevesy (17) on nucleic acid turnover allows such an approximation.

As far as absolute magnitudes of turnover are concerned the data on phospholipid turnover in the cell-free system for instance are quite comparable with the known rate in intact rat liver slices. Fishler *et al.* (18) calculated that at a 1 hour interval an average of 1.4  $\gamma$  of inorganic P and at a 4 hour interval 10.8  $\gamma$  was incorporated into phospholipid per gm. of wet tissue when 300 mg. of rat liver slices were suspended in 5.0 ml. of buffer solution containing 180  $\gamma$  of labeled inorganic P. They were not able to demonstrate an incorporation with homogenates. In the present investigation with a properly supplemented cell-free enzyme suspension, the incorporation of  $P^{32}$  into phospholipid, if calculated on the basis used by the California group, amounts to at least 1.0  $\gamma$  of P per gm. of wet tissue at a 20 minute interval, a somewhat greater rate than was observed in the intact slice. The data in this study are consistent with the findings of Chaikoff's group (1) that phospholipid formation is dependent upon phosphate exchanges coupled to oxidation. The use of the supplemented cell-free system described here should make possible further studies on factors governing phospholipid formation, in extension of Chaikoff's work.

The relatively rapid turnover of "phosphoprotein" phosphorus found corroborates the data of Brues *et al.* (19), as originally interpreted by Spiegelman and Kamen (20), which indicate that a non-nucleic acid phosphorus component of unknown identity is involved in phosphate exchanges in the acid-insoluble esters of rat liver. Furthermore, these findings are also reminiscent of the data of Juni *et al.* (21) and of Spiegelman and Kamen (20), demonstrating that a metaphosphate complex found in a corresponding fraction of yeast cells has an extremely high rate of turnover. In the cell-free system in this study, the unidentified residue possessed the highest specific activity of the acid-insoluble esters and this turnover was in turn coupled to oxidation. No intensive study of the identity of this fraction was made. It does not appear to have the gross properties of the active yeast metaphosphate.

#### SUMMARY

In a washed cell-free suspension of the particulate material of rat liver, consisting largely of nuclei and mitochondria, oxidation of *l*-malate over the Krebs tricarboxylic acid cycle in the presence of inorganic phosphate

labeled with P<sup>32</sup>, adenine nucleotide, and Mg<sup>++</sup> leads to incorporation of P<sup>32</sup> into fractions of the particulate material corresponding to phospholipides, pentose nucleic acid, and an unidentified residue of "phosphoprotein." Incorporation into the desoxypentose nucleic acid fraction was negligible. In the absence of active oxidation and phosphorylation of the adenylic acid system there was little or no incorporation into any fraction. The rate of incorporation of the P<sup>32</sup> into the acid-insoluble esters was much lower than into the acid-soluble fraction, as is known to be the case in intact tissues.

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# EFFECT OF NICOTINAMIDE ON BLOOD SUGAR AND BLOOD ACETONE BODIES OF DIABETIC AND NORMAL SUBJECTS

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(Received for publication, February 3, 1948)

Gobell (1) showed that 100 mg. of nicotinamide injected intravenously into children produced regularly a fall in the blood sugar level, averaging 21.3 mg. per cent. The hypoglycemic action of nicotinic acid was also observed in normal human adults by Marche and Delbarre (2), Poumeau-Delille and Fabiani (3), and Neuwahl (4). In experiments with animals Ledrut (5) observed a marked decrease in the blood sugar level in normal rabbits which received nicotinic acid orally or intravenously. Large doses of nicotinic acid injected into rats produced hypoglycemia in the hands of Cherkes and Rozenfeld (6). Neuwahl reported (4) that an intravenous injection of 250 mg. of nicotinic acid in eight non-diabetic subjects produced a blood sugar level as low as 48 mg. per 100 cc., which led to long lasting rigor. When glucose was injected along with nicotinic acid, no reaction was observed. Horst (7), on the other hand, reported that the administration of nicotinamide to persons with normal metabolism had no influence on the fasting blood sugar level or on the glucose tolerance curve. Neuwahl (4) observed a definite improvement in the carbohydrate tolerance in twelve diabetic patients receiving nicotinic acid. Gordon (8) reported a patient on a diet of 1200 calories who had a fasting blood sugar of 160 mg. per 100 cc. of blood. After the patient was fed 1200 mg. of nicotinamide per day for 1 month, the blood sugar dropped to 60 mg. per 100 cc. of blood. Contrary to the findings of Neuwahl (4) and Gordon (8), Poumeau-Delille and Fabiani (3) did not notice any effect of nicotinic acid on the glycemia of diabetic patients. Wade (9) also reported that 600 mg. of nicotinamide fed thrice daily for 14 days to six diabetic patients who were receiving adequate insulin neither influenced the sugar tolerance nor improved the diabetic condition.

In view of the conflicting reports cited above and in view of the observation that nicotinic acid prevents the diabetogenic action of alloxan (10) in rabbits and rats, it was thought desirable to study the effect of intravenous injection of nicotinamide on the blood sugar and blood acetone bodies of diabetic and normal subjects.

## EXPERIMENTAL

*Effect of Nicotinamide on Blood Sugar of Diabetic and Normal Subjects—*500 mg. of nicotinamide in a 10 per cent solution were injected intravenously

into seventeen diabetic and six normal subjects which had fasted overnight. Samples of blood were taken before and at intervals of half an hour up to 2 hours after the injection. Blood sugar was determined by the method of Hagedorn and Jensen (11). The results are given in Table I.

TABLE I  
*Effect of Intravenous Injection of 500 Mg. of Nicotinamide on Blood Sugar of Diabetic and Normal Subjects*

Subject	Age	Fasting blood sugar	Blood sugar after injection of nicotinamide			
			$\frac{1}{2}$ hr.	1 hr.	1½ hrs.	2 hrs.
	yrs.	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
<b>Diabetic</b>						
S. K. M. ....	70	180	200	189	195	199
J. C. B. ....	48	286	278	230	260	216
S. B. ....	28	521	527	514	534	550
S. N. B. ....	70	117	127	120	117	117
S. C. G. ....	60	138	143	140	143	143
B. S. ....	42	385	399	390	340	333
F. R. ....	27	110	110	110	116	134
S. S. ....	40	186	213	226	228	260
R. K. G. ....	53	251	270	245	232	257
R. B. S. ....	37	241	247	262	276	276
N. M. ....	16	324	326		328	297
M. N. M. ....	40	230	211	186	202	179
M. P. ....	37	236	226	232	222	221
N. A. ....	70	298	282	278	292	316
D. M. K. ....	44	288	290	278	264	280
S. S. ....	39	270	270	270	284	274
K. C. D. ....	37	224	224	217	195	195
Average .....		252 ± 24	255 ± 24	243 ± 24	248 ± 23	250 ± 23
<b>Normal</b>						
S. C. A. ....	45	100	100	103	96	105
A. M. K. ....	47	93	93	93	97	93
M. L. ....	30	97	110	117	115	122
M. J. ....	45	109	117	126	117	112
H. M. ....	41	92	88	93	95	84
S. N. M. ....	41	100	87	100	87	87
Average .....		99	99	105	101	100

*Effect of Nicotinamide on Blood Acetone Bodies of Diabetic and Normal Subjects*—500 mg. of nicotinamide in a 10 per cent solution were injected intravenously into ten diabetic and three normal subjects which had fasted overnight. Samples of blood were taken as before. Total acetone bodies

of blood were determined as follows: 1 cc. of oxalated blood was delivered into 10 cc. of distilled water taken in a 25 cc. stoppered measuring cylinder. After the laking was complete, 2 cc. of 0.3 N barium hydroxide were added and the contents well mixed by shaking. 2 cc. of a 5 per cent solution of zinc sulfate were then added. The contents were mixed and centrifuged. 10 cc. of the centrifugate were then transferred to an all-glass distilling

TABLE II  
*Effect of Intravenous Injection of 500 Mg. of Nicotinamide on Acetone Bodies of Blood of Normal and Diabetic Subjects*

Subject	Age	Fasting blood acetone bodies	Blood acetone bodies after injection of nicotinamide			
			$\frac{1}{2}$ hr.	1 hr.	1½ hrs.	2 hrs.
	yrs.	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
<b>Diabetic</b>						
S. K. M. ....	70	1.64	1.64		4.0	16.8
J. C. B. ....	48	1.46	1.34		1.12	0.88
S. B. ....	28	4.50	4.52		2.53	2.25
S. N. B. ....	70	1.03		0.50	0.54	
S. C. G. ....	60	1.88	1.60	1.12	1.06	1.30
B. S. ....	42	1.36	1.10	3.77		3.45
S. S. ....	40	4.60	4.00		8.00	10.32
J. C. D. ....	37	1.12	1.10	1.04	1.10	1.08
J. N. S. ....	53	1.10	1.34	1.48	1.23	1.06
K. C. D. ....	37	41.80	55.80	56.00	56.40	40.00
Average .....		6.01 $\pm$ 4	8.05 $\pm$ 6	10.65 $\pm$ 9	8.50 $\pm$ 6	8.57 $\pm$ 4
<b>Normal</b>						
S. C. A. ....	45	0.95	0.96		3.00	1.46
A. M. K. ....	47	0.59	0.64	0.60	0.64	
R. F. ....	26	1.20	1.20	1.31	1.75	2.77
Average .....		0.91	0.93	0.95	1.13	1.41

apparatus with only one ground glass joint and 10 cc. of distilled water were added. The distilling flask was heated on a micro burner. 0.46 per cent potassium dichromate in 15.6 N sulfuric acid was added drop by drop, from a dropping funnel attached to the middle of the neck of the flask, when the contents of the flask began to boil. The distillation was continued for 20 minutes, and the distillate was collected into a conical flask containing 2 cc. of distilled water. An aliquot of the distillate was allowed to react with 2,4-dinitrophenylhydrazine and the total acetone bodies were estimated as described by Greenberg and Lester (12). The results are given in Table II.

From the results it will be seen that nicotinamide had practically no effect on the blood sugar and blood acetone bodies of diabetic and normal subjects. The claim of Gobell (1), therefore, could not be confirmed.

#### SUMMARY

The effect of intravenous injection of 500 mg. of nicotinamide on the blood sugar and blood acetone bodies of diabetic and normal subjects has been studied.

The blood sugar and the blood acetone bodies are practically unaffected by the injection of nicotinamide in both diabetic and normal subjects.

The authors are indebted to N. P. Chatterjee of the East India Pharmaceutical Works, Calcutta, for the nicotinamide and to the Indian Research Fund Association for a research grant.

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# THE SPECIFIC PEPTIDASE AND ESTERASE ACTIVITIES OF CHYMOTRYPSIN\*

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(Received for publication, September 20, 1948)

The experiments of Bergmann and Fruton on the substrate specificity of crystalline chymotrypsin demonstrate that this enzyme catalyzes the hydrolysis of tyrosyl and phenylalanyl peptides at the peptide bond which involves the carbonyl group of these aromatic amino acid residues (2-4). Typical substrates are N-substituted L-tyrosylglycinamides which are split into N-substituted L-tyrosine and glycineamide. Representative examples of this type of substrate are benzoyl-L-tyrosylglycinamide, carbobenzoxy-L-tyrosylglycinamide, and glycyl-L-tyrosylglycinamide. The proteolytic coefficients for the hydrolysis of these substrates in 0.05 M substrate concentration are of the order of  $1 \times 10^{-2}$  to  $1 \times 10^{-3}$ . Whereas in these peptides the susceptible peptide bond occupies an internal position, substrates containing the susceptible bond in a terminal position (amides) are likewise hydrolyzed by chymotrypsin. A representative example of this type of substrate is glycyl-L-tyrosinamide, which is split into glycyl-L-tyrosine and ammonia, the proteolytic coefficient being about  $6 \times 10^{-3}$  (4). Phenylalanyl analogues of these substrates are hydrolyzed at a considerably lower rate.

According to the published data (2-4), it is irrelevant for hydrolysis whether the N-substituted L-tyrosylglycinamides have a free or masked terminal amino group. Thus, glycyl-L-tyrosylglycinamide and its phenylalanine analogue, which contain a positively charged amino group, are typical substrates, as are the uncharged molecules, benzoyl-L-tyrosylglycinamide and carbobenzoxy-L-tyrosylglycinamide. However, it has been reported that masking of the free amino group of glycyl-L-tyrosinamide by a carbobenzoxy (4) group practically eliminates the hydrolysis of the resulting peptide by chymotrypsin. Analogously, while L-tyrosinamide and L-phenylalaninamide are slowly hydrolyzed by this enzyme, at a rate approximately one-twentieth of that of the corresponding N-glycyl derivatives (4), benzoyl- (3) or carbobenzoxy-L-tyrosinamide (4) and their respec-

\* Part of a thesis to be submitted by Mr. Seymour Kaufman to the Graduate School of Duke University in partial fulfillment of the requirements for the degree of Doctor of Philosophy. A preliminary account of this work has already been published (1).

tive L-phenylalanine analogues have been reported to be entirely resistant to enzymatic splitting (4). Since chymotryptic activity is allegedly directed toward the peptide group which involves the carbonyl group of the aromatic amino acid residues, regardless of whether it occupies an internal or terminal position, it appears that, contrary to the published data, N-substituted tyrosinamides should be typical substrates for this enzyme. The results of the present investigation substantiate this prediction, and, moreover, show that, in aqueous methanol, N-acylated derivatives of L-tyrosinamide are hydrolyzed more rapidly than are any of the substrates previously described by Bergmann and Fruton.

Because of the very limited kinetic data that are available for the peptide hydrolysis by chymotrypsin, a comprehensive investigation of the kinetics of hydrolysis of the typical substrate, glycyl-L-tyrosinamide, and of the new substrate described herein, *i.e.* benzoyl-L-tyrosinamide, was undertaken. This included determination of the influence of substrate concentration on reaction kinetics (5), determination of the enzyme-substrate dissociation constant,  $K_m$ , and for representative peptide and ester (*vide infra*) substrates, determination of the energetic constants of the reaction. Preliminary data on the influence of pH on hydrolysis rates are likewise included in this paper.

In a previous report from this laboratory on the specific esterase activity of trypsin (6), preliminary data on a like specificity of chymotrypsin were included. Since then, a specific esterase activity of another proteolytic enzyme, *i.e.* crystalline carboxypeptidase, has been established (7). As a logical sequence to these studies, the specific esterase activity of chymotrypsin has been investigated in greater detail. The most active, specific, esters which so far have been found are benzoyl-L-tyrosine ethyl ester and acetyl-L-tyrosine ethyl ester. Quantitative measurements on the kinetics and energetics of the hydrolysis of these esters have been made and compared to analogous data for the hydrolysis of one of the parent peptides. A search for specific inhibitors for chymotrypsin has been in progress. Preliminary experiments are included in this report, although they yielded negative results.

#### EXPERIMENTAL

*Enzymes and Methods*—The preparation of chymotrypsin and the methods for measuring amidase and esterase activities have already been described (6). Enzyme and substrate solutions were freshly prepared for each experiment. Unless otherwise stated, measurements were performed in the presence of phosphate buffer, pH 7.8, at 25°. Buffer concentration was 0.1 M in the absence of methanol, and 0.045 to 0.050 M in measurements carried out in the presence of methanol.

*Substrates*—Glycyl-L-tyrosinamide acetate (GTA) was prepared<sup>1</sup> essentially according to the directions of Fruton and Bergmann (4). Reduction of carbobenzoxyglycyl-L-tyrosinamide by catalytic hydrogenation in the presence of acetic acid (4) yielded only small amounts of GTA. Extensive experimentation showed that good yields of GTA could be obtained if the acid was withheld till after completion of the catalytic reduction. After conversion to the acetate salt and recrystallization (4), yields of 75 per cent were obtained.

Calculated, N 14.1; found, N 14.2

Benzoyl-L-tyrosinamide (BTA) was synthesized according to Bergmann and Fruton (3). It was recrystallized twice from absolute ethanol. M.p. found, 210–211°; reported (3), 198–199°.

Calculated, N 9.9; found, N 9.8

Acetyl-L-tyrosine ethyl ester (ATEE). Acetyl-L-tyrosine was prepared according to du Vigneaud's method (8). Esterification was carried out in the usual manner. The ester was recrystallized from aqueous methanol. M.p. 79–80°.

$C_{13}H_{17}NO_4$  (251.0). Calculated. C 62.08, H 6.78, N 5.48  
Found. " 62.07, " 6.75, " 5.45

Benzoyl-L-tyrosine ethyl ester (BTEE) was prepared from benzoyl-L-tyrosine (3) in the usual manner and recrystallized twice from aqueous ethanol. M.p. 120–121°; mixed melting point with benzoic acid, 90–93°.

Calculated, N 4.4; found, N 4.3

1-Phenyl-2-acetaminobutanone-3 (PAAB) was prepared according to the directions of Levene (9). It was recrystallized twice from toluene. M.p. found, 96–97°; reported (9), 98–99°.

Calculated, N 6.8; found, N 6.6

Benzoyl-L-tyrosine hydrazide (BTH) was synthesized according to Bergmann and Fruton (3). M.p. found, 251–253°; reported (3), 250–255°.

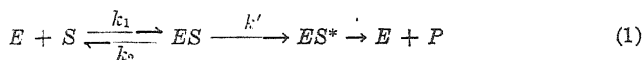
## Results

### Reaction Kinetics

It has already been shown (5) that interpretation of kinetic data of hydrolysis by proteolytic enzymes by first order reaction kinetics is of limited validity, the shift in equilibrium between free and combined enzyme during the course of the reaction causing marked deviations from the first order equation.

<sup>1</sup> This substrate was prepared by Mr. John E. Snoke of this laboratory.

If the reaction is pictured as occurring in three steps, of which the first is a reversible combination of enzyme  $E$  and substrate  $S$  to form the complex  $ES$



where  $e$  is the total enzyme concentration,  $p$  the concentration of the complex  $ES$ , and  $a$  the concentration of the substrate, then the velocity of the forward reaction is given by

$$v_1 = k_1 (e - p)(a) \quad (2)$$

and that of the reverse reaction by

$$v_2 = k_2 (p) \quad (3)$$

$ES$  then passes to an activated state, corresponding to that of the highest energy barrier, denoted by  $ES^*$ , at a rate given by

$$v' = k' (p) \quad (4)$$

and decomposes subsequently into free enzyme and reaction products,  $P$ . Although theory requires that the activation and decomposition reactions be considered as reversible, for purposes of simplification, only the experimentally measurable steps shown here have been considered.

Since at equilibrium

$$v_1 = v_2 + v' \quad (5)$$

it follows that

$$K_m = \frac{(e - p)(a)}{(p)} = \frac{k_2 + k'}{k_1} \quad (6)$$

The rate of disappearance of the substrate is given by

$$v' = k'(p) = -\frac{da}{dt} = \frac{k'ea}{K_m + a} \quad (7)$$

which on integration yields

$$k'et = 2.3 K_m \log \frac{a_0}{a} + (a_0 - a) \quad (8)$$

Here  $e$  is the total enzyme concentration in mg. of enzyme N per cc.,  $t$  is the time in minutes,  $a_0$  is the initial substrate concentration, and  $a$  the substrate concentration at time  $t$ , the latter two in moles per liter.

Since the specific constant,  $k'$ , characterizing the rate of disappearance of the substrate (equation (8)), is given by the sum of a first order term and of a zero order term, it is evident that the reaction will approach first



order kinetics as  $K_m$  increases, or specifically, as  $k_1$  decreases in comparison to  $k_2 + k'$  (equation (6)). Conversely, the reaction course will approach zero order kinetics as  $K_m$  decreases, or specifically, as  $k_1$  increases in comparison to  $k_2 + k'$ .

Since it was found in the present work that, with all substrates that were tested, the apparent first order reaction constant decreased with increasing substrate concentration, equation (8) was used to evaluate reaction rates. The value of  $K_m$  used in this equation was determined from the relation of Lineweaver and Burk (10) as previously described (5).

For comparison with the work of Bergmann and Fruton (2-4), apparent proteolytic coefficients were likewise calculated from the initial, linear portions of the curves obtained when the experimental data were plotted according to the first order equation. Maximum proteolytic coefficients,  $C_{\max.}$ , were calculated as previously described (5), with use of the relation<sup>2</sup>

$$C_{\max.} = \frac{1}{2.3} \frac{k'}{K_m} \quad (9)$$

#### Peptidase Activity

*Glycyl-L-tyrosinamide Acetate (GTA)*—The enzymatic activity of the preparation of chymotrypsin was tested against GTA, for which quantitative data are available in the literature (4). In 0.05 M substrate solution, 0.1 M phosphate buffer, pH 7.8, 25°, the apparent proteolytic coefficient<sup>3</sup> was  $C = k/e = 0.0097 \pm 0.0011$  (five determinations at varying enzyme concentrations). This value is significantly higher than that obtained by Fruton and Bergmann under similar experimental conditions,<sup>4</sup> i.e.  $C = 0.0065$ .

The apparent proteolytic coefficient increased with decreasing substrate concentration,  $C$  being  $0.0121 \pm 0.0016$  in 0.025 M concentration of GTA, and 0.0129 in 0.0125 M concentration of this substrate.  $K_m$  was deter-

<sup>2</sup> This equation represents an approximation, since it presupposes first order reaction kinetics to apply as substrate concentration approaches zero. It is valid only if  $k_2$ , the specific reaction constant for the dissociation of the enzyme-substrate complex, is negligibly small in comparison to  $k'$ , since  $K_m = (k_2 + k')/k_1$  (see also footnote 1 of reference (5)).

<sup>3</sup>  $k$  denotes the first order reaction constant as defined in Bergmann's work, i.e.  $k = (1/t) \log_{10} a_0/a$ , where  $a_0$  and  $a$  denote, respectively, initial substrate concentration and substrate concentration at time  $t$ .

<sup>4</sup> It was found that GTA was spontaneously hydrolyzed at pH 7.8, even when stored at 4°. The extent of spontaneous hydrolysis was negligible during the experimental period of 2 to 3 hours; however, blank corrections were made in each enzymatic experiment. The crystals which were collected after storage of a stock solution of GTA in the cold, melted with decomposition at 265-275°. Abderhalden (11) reports a melting point of 295° for glycyl-L-tyrosine.

mined from a plot according to equation (15) of reference (5) where  $v$  was taken as the moles per liter of substrate hydrolyzed during the first 5 minutes of the reaction. This plot is shown in Fig. 1 together with similar plots for other substrates.  $K_m$  was calculated as 0.122. Substitution of this value into equation (8) yielded for each rate experiment a linear relation from which  $k'$  was determined to be  $4.1$  to  $4.3 \times 10^{-3}$  when  $a_0$  was varied from 0.05 M to 0.0125 M. Substitution of these values into equation (9) yielded  $C_{\max.} = 0.015$ .

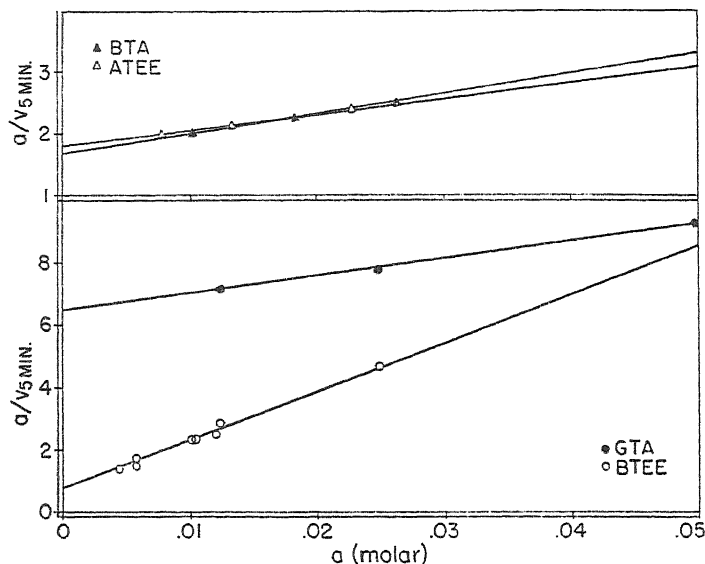


FIG. 1. A plot of the Lineweaver-Burk (10) equation for the determination of enzyme-substrate dissociation constants,  $K_m$ . The curves refer to the substrates indicated; for details, see the text and Table I.

Since all of the other substrates that were studied in this work were relatively insoluble in water but soluble in dilute methanol, the rate of enzymatic hydrolysis of GTA was measured as a function of the methanol concentration, in order to provide a common basis of comparison of hydrolysis rates. It was found that in 0.025 M solutions of GTA the apparent proteolytic coefficient decreased logarithmically with increasing methanol concentration, as shown in Fig. 2, along with similar data for BTEE. The slopes of the straight lines were practically identical for the peptide (GTA) and for the ester (BTEE), *i.e.* 0.0279 for the former and 0.0276 for the latter. Since the presence of methanol introduces a constant second order term, it appears that the activity of chymotrypsin is essentially undiminished. This finding is in qualitative agreement with the work

of Risley *et al.* (12) in which it was found that trypsin exhibits considerable activity in ethanol solutions up to 30 per cent. The quantitative relation established by the present measurements renders it possible to compare the hydrolysis rates of substrates, which are relatively insoluble in water but soluble in methanol-water mixtures, with the hydrolysis rate of a "standard" water-soluble substrate for chymotrypsin, *i.e.* GTA.

*Benzoyl-L-tyrosinamide (BTA)*—BTA, previously reported to be entirely resistant to chymotryptic hydrolysis (3), was found to be hydrolyzed

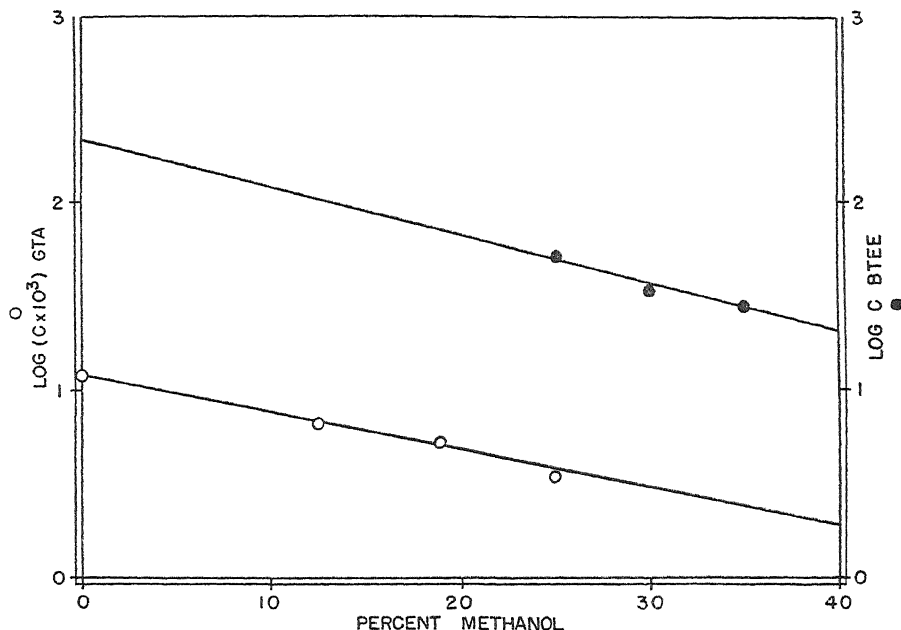


FIG. 2. Variations of apparent proteolytic coefficients,  $C$ , for the hydrolysis of 0.025 M GTA (○) and of 0.0128 M BTEE (●) with methanol concentrations. The slopes of the straight lines are  $-0.0279$  for GTA, and  $-0.0276$  for BTEE.

more rapidly than GTA. Because of its limited solubility in water, measurements were performed in 30 per cent methanol, 0.05 M phosphate buffer, pH 7.8. In 0.0266 M substrate solutions, the apparent proteolytic coefficient was  $C = 0.0453$  or about 20 times higher than the value obtained for GTA by extrapolation of the data plotted in Fig. 2 to 30 per cent methanol ( $C = 0.0023$ ). The apparent proteolytic coefficient increased with decreasing substrate concentration, being  $C = 0.0498$  in 0.0186 M substrate solution, and  $C = 0.0597$  in 0.0106 M substrate solution.  $K_m$ , determined from the data plotted in Fig. 1, was 0.0424. Substitution of this value into equation (8) gave values of  $k' = 6.47 \pm 0.31 \times 10^{-3}$  (six

measurements at varying enzyme and substrate concentrations). From these values,  $C_{\max.}$  was calculated as 0.066.

### *Esterase Activity*

*Benzoyl-L-tyrosine Ethyl Ester (BTEE)*—This specific ester was hydrolyzed by chymotrypsin considerably faster than any of the specific peptides previously described. Hydrolysis apparently followed first order reaction kinetics; however, an unusually large substrate concentration dependence of the apparent proteolytic coefficients was noted. Because of the uncertainty of the initial substrate concentration in potentiometric measurements of esterase activity (6), recourse was had to the determination of  $a_0$  by interpolation according to a method previously described (6). It follows from the properties of the equation of first order reaction kinetics that for any consecutive pair of time values, such that  $t_2 = 2t_1$ ,

$$a_0 = x_2^2 / (2x_1 - x_2) \quad (10)$$

where  $x_2$  and  $x_1$  are the moles per liter of substrate hydrolyzed at times  $t_2$  and  $t_1$ . In order to test the accuracy of this method on substrates for chymotrypsin, the rate of hydrolysis of BTA by chymotrypsin was determined, with the use of an initial, analytically determined, substrate concentration of 0.025 M. The initial substrate concentration was also calculated from various portions of the curve shown in Fig. 3, with the results given in the legend to Fig. 3. It will be noted that, within the region corresponding to 43 to 88 per cent of hydrolysis, the calculated value of  $a_0$  agrees within 3 per cent with the analytical value.

When this method of calculation was applied to the kinetics of esterase activity of chymotrypsin, the apparent proteolytic coefficient for the hydrolysis of BTEE was found to vary from about  $C = 18$  in 0.025 M initial substrate concentration to about  $C = 83$  in 0.005 M initial substrate concentration, as shown in the upper curve of Fig. 4. The lower of these two values is still some 8000 times higher than the extrapolated value for the same concentration of GTA in 30 per cent methanol ( $C = 0.0023$ ). From the plot shown in Fig. 1,  $K_m$  was determined as 0.0039. Substitution of this value into equation (8) yielded  $k' = 8.15 \pm 0.57 \times 10^{-1}$  (eight determinations at varying enzyme and substrate concentrations). The relative constancy of  $k'$  over the same range of substrate concentration in which  $C$  varied about  $4\frac{1}{2}$ -fold is shown in Fig. 4.  $C_{\max.}$  (calculated according to equation (9)) was 91.

*Acetyl-L-tyrosine Ethyl Ester (ATEE)*—A relatively small dependence of the apparent proteolytic coefficient on initial substrate concentration was noted,  $C$  being 20.3, 23.7, and 25.3, respectively, for substrate concentrations of 0.023, 0.0136, and 0.008 M. From the plot shown in Fig. 1,

$K_m$  was calculated as 0.074. Substitution of this value into equation (8) yielded a value of  $k' = 4.49 \pm 0.12$  (three determinations). The strict adherence of the hydrolysis rate to equation (8) is shown for a typical experiment in Fig. 5 in which the right-hand side of equation (8) is plotted

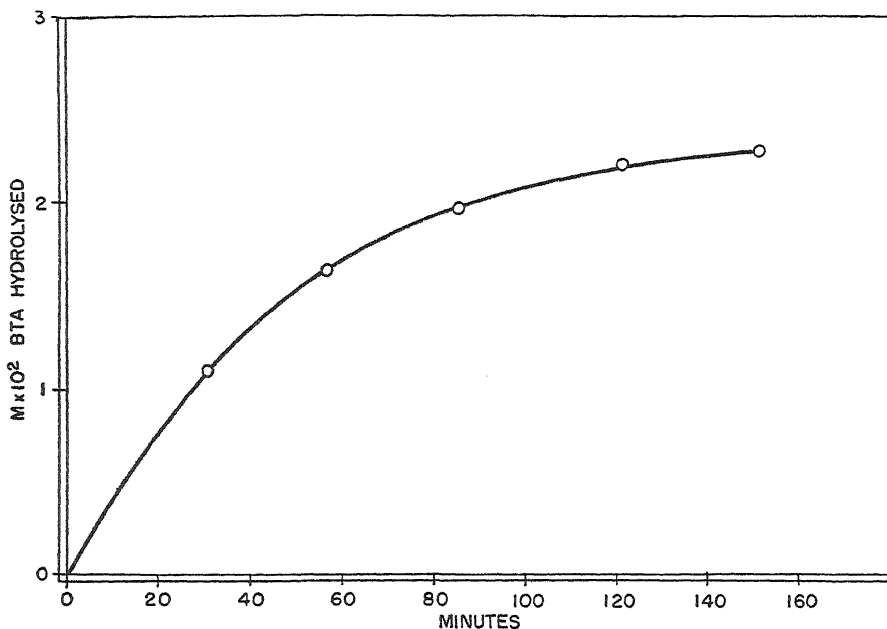


FIG. 3. Rate of hydrolysis of BTA by chymotrypsin. The analytical, initial substrate concentration  $a_0$  was 0.025 M. Calculations of  $a_0$  by equation (10) of the text gave the following values:

$t_1$	$t_2$	Range of hydrolysis	$a_0$	$t_1$	$t_2$	Range of hydrolysis	$a_0$
min.	min.	per cent	M	min.	min.	per cent	M
20	40	16-52	0.0270	50	100	61-83	0.0243
30	60	43-67	0.0248	60	120	67-88	0.0244
40	80	52-76	0.0242	70	140	72-90	0.0239

against time.<sup>5</sup> The relation between initial substrate concentration and  $C$  and  $k'$ , respectively, is shown in Fig. 6, which demonstrates the constancy of  $k'$  over the same range in which  $C$  markedly increases.  $C_{\max.}$  (equation (9)) was calculated as 26, which is close to the value of  $C$  determined from the lowest substrate concentration. A summary of all

<sup>5</sup> Similar adherence to equation (8) was found for all substrates that were studied in this work.

kinetic constants determined at 25° for the two peptide substrates and the two ester substrates is given in Table I. Interpretation of these data will be given in the discussion of this paper.

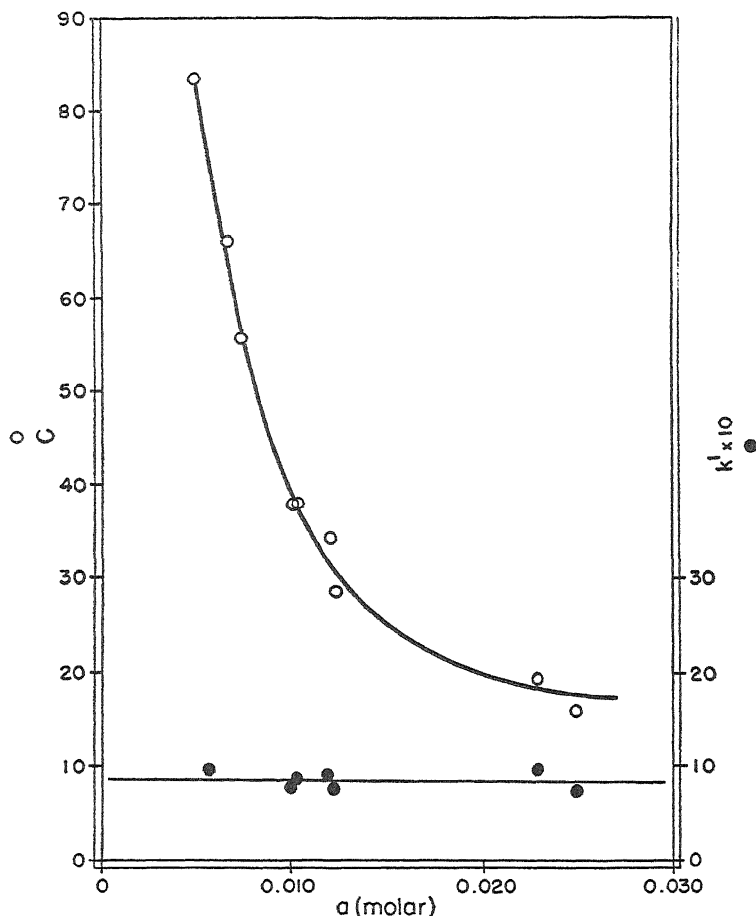


FIG. 4. A plot demonstrating the large substrate concentration dependence of apparent proteolytic coefficients,  $C$ , for the hydrolysis of BTEE by chymotrypsin, as compared to the substrate concentration independence of the specific reaction constant  $k'$  (calculated from equation (8)). For further details, see the text and Table I.

#### *Influence of Temperature on Reaction Rates*

According to the concept of Michaelis and Menten (13) it has been assumed in this work that enzymatic hydrolysis proceeds in at least two steps: The first of these is the formation of a stable enzyme-substrate

complex, governed by the affinity constant, or its inverse function, the dissociation constant,  $K_m$ . The second step involves the activation of the stable complex and subsequent decomposition into the reaction products and free enzyme, and is governed by the specific reaction constant,  $k'$ .<sup>6</sup>

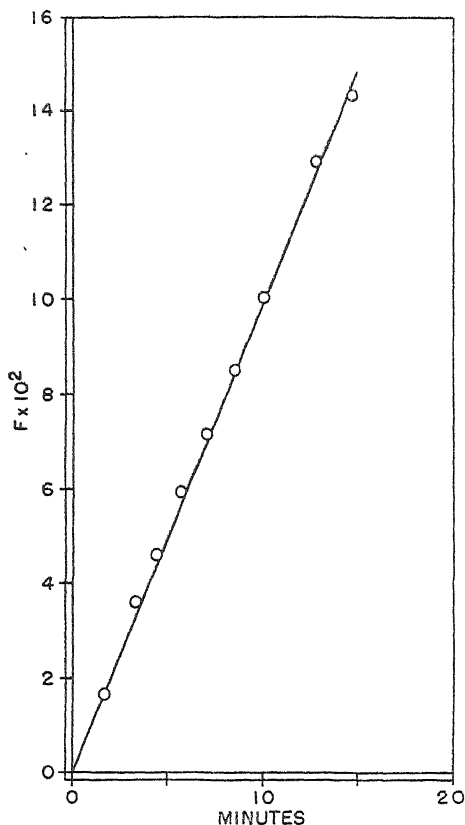


FIG. 5. The rate of hydrolysis of ATEE by chymotrypsin, calculated according to equation (8).  $F$  denotes the right-hand side expression of equation (8), and is plotted along the axis of the ordinate against time, which is plotted along the axis of the abscissa. The slope of the straight line is equal to  $k'e$ .

Since  $K_m$  defines a state of equilibrium, determination of the influence of temperature on  $K_m$  yields the classical thermodynamic constants,  $\Delta H$ ,  $\Delta F$ , and  $\Delta S$ , the heat of the reaction, the free energy, and the entropy

<sup>6</sup> Since  $K_m$  as defined herein (equation (6)) contains explicitly  $k'$ , its inverse function is not an exclusive measure of the affinity of the enzyme for the substrate. The true affinity constant,  $k_1/k_2$ , is indeterminate in these as in any kinetic studies which are based on measurements of the rate of disappearance of the substrate.

of the reaction, respectively. The following relations have been used to evaluate these constants.

$$\log \frac{K_m''}{K_m'} = \frac{\Delta H}{2.3R} \left( \frac{1}{T_1} - \frac{1}{T_2} \right) \quad (11)$$

$$\Delta F = -2.3RT \log K_m \quad (12)$$

$$\Delta S = \frac{1}{T} (\Delta H - \Delta F) \quad (13)$$

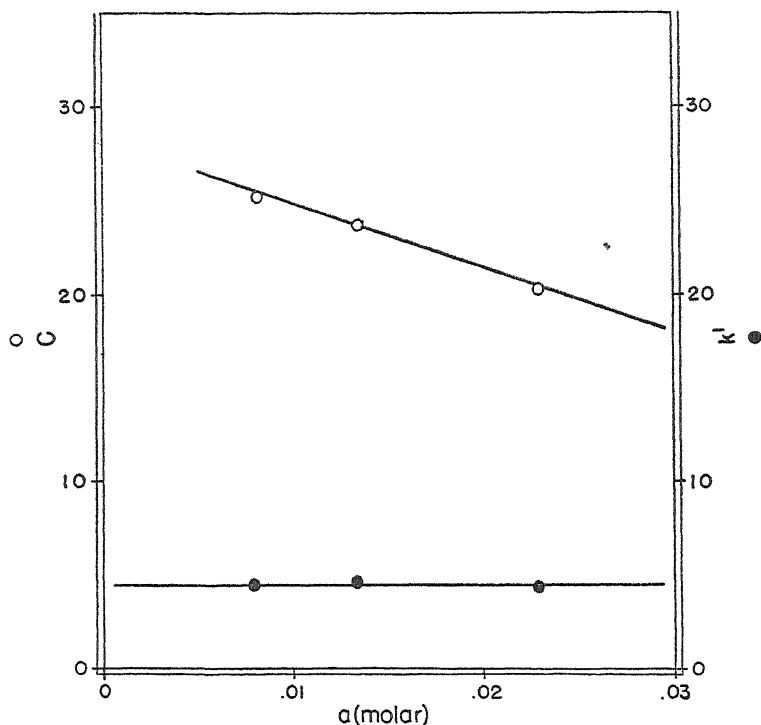


FIG. 6. A plot showing the substrate concentration dependence of the apparent proteolytic coefficient,  $C$ , for the hydrolysis of ATEE by chymotrypsin, as compared to the substrate concentration independence of the specific reaction constant,  $k'$  (calculated from equation (8)). For further details see the text and Table I.

Calculations of  $\Delta F$  and  $\Delta S$  have been referred to a standard temperature of 298° K.

The relation between the specific reaction constant,  $k'$ , and temperature may be formally expressed by the Arrhenius equation

$$\log \frac{k'_2}{k'_1} = \frac{\Delta E}{2.3R} \left( \frac{1}{T_1} - \frac{1}{T_2} \right) \quad (14)$$



where  $\Delta E$  denotes the critical increment of temperature ( $\Delta E = \Delta H^* + RT$ ). From this value, the heat of activation,  $\Delta H^*$ , and the free energy,  $\Delta F^*$ , and the entropy of activation,  $\Delta S^*$ , may be determined (14) by the equations

$$k'_0 = \frac{kT}{h} \exp \frac{-\Delta H^*}{RT} \exp \frac{\Delta S^*}{R} = 6.25 \times 10^{12} \exp \frac{-\Delta H^*}{RT} \exp \frac{\Delta S^*}{R} \quad (15)$$

$$\Delta F^* = \Delta H^* - T\Delta S^* \quad (16)$$

TABLE I

*Kinetic Constants for Hydrolysis of Specific Peptide and Ester Substrates by Chymotrypsin at pH 7.8, 25°*

$a_0$  is the initial substrate concentration;  $C$  is the proteolytic coefficient calculated on the assumption of first order reaction kinetics,  $C_{\max}$  is the maximum proteolytic coefficient calculated from equation (9). For the determinations of  $K_m$  and  $k'$ , see the text.

Substrate	Methanol	$a_0$	$C$	$C_{\max}$	$K_m$	$k'$
	<i>per cent</i>	$10^{-2} M$				
GTA	0	5.00	0.0097	0.015	0.122	0.0041
	0	2.50	0.0121			
	0	1.25	0.0129			
	30*	2.50	0.0023			
BTA	30	2.66	0.0453	0.066	0.0424	0.0065
	30	1.86	0.0498			
	30	1.06	0.0597			
BTEE	30	2.50	18.0	91	0.0039	0.82
	30	1.00	38.0			
	30	0.50	83.5			
ATEE	30	2.30	20.3	26	0.074	4.5
	30	1.36	23.7			
	30	0.80	25.3			

\* By extrapolation, by means of the equation,  $\log (C \times 10^3) = 1.15 - 0.0276$  (per cent methanol). For details, see the text and Fig. 2.

where  $k$  denotes the Boltzmann constant,  $h$ , the Planck constant,  $R$ , the gas constant, and  $k'_0$  the absolute specific reaction constant<sup>7</sup> in sec.<sup>-1</sup>, corrected to an enzyme concentration of 1 mole per liter (molecular weight of chymotrypsin assumed to be 36,000), at a standard temperature of 298° K.

Measurements of the effects of temperature on  $K_m$  and  $k'$  have been carried out with the substrates BTA and BTEE. The temperatures were 9.5°, 18°, and 25° for BTA, and 7.5°, 15.5°, and 25° for BTEE. All

<sup>7</sup> Since  $k'$  is the specific reaction constant per mg. of enzyme N per cc., per minute, the relation between  $k'_0$  and  $k'$  is simply  $k'_0 = (k'/60) (36,000/6.25)$ , assuming a molecular weight for chymotrypsin of 36,000.

measurements were performed in the presence of 30 per cent methanol, and 0.05 phosphate buffer, pH 7.8. The results are shown in Fig. 7 in which  $\log k'$  and  $\log K_m$ , respectively, are plotted along the axis of ordinates and  $1/T \times 10^3$  along the axis of abscissas. From these sets of data, energetic constants were calculated by equations (11) to (16), and

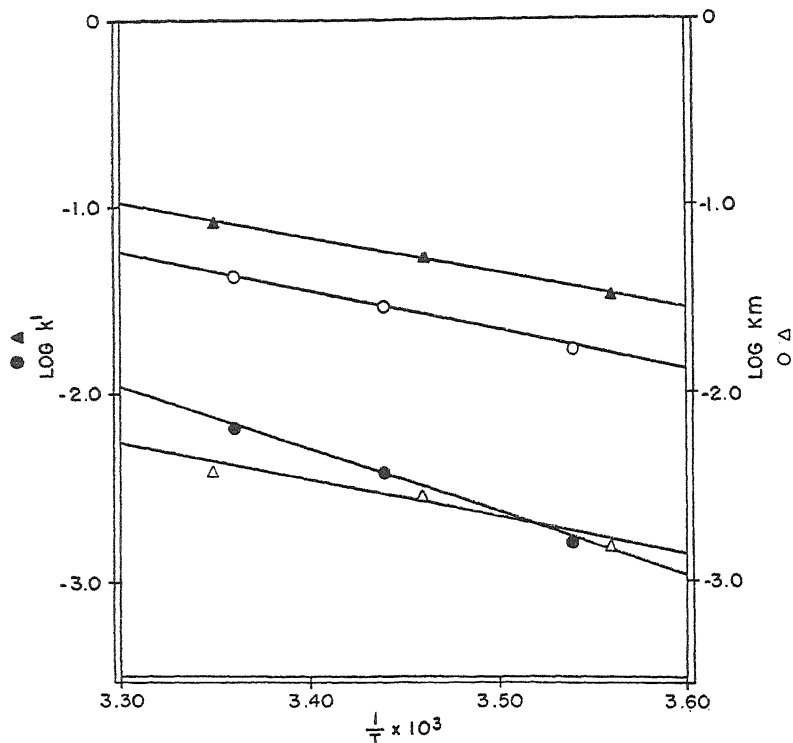


Fig. 7. A plot of the logarithm of  $K_m$  ( $\circ$  and  $\triangle$ ) and of the logarithm of  $k'$  ( $\bullet$  and  $\blacktriangle$ ) against reciprocal temperatures for the calculation of heats of reaction and critical temperature increments for the chymotryptic hydrolysis of BTA ( $\circ$  and  $\bullet$ ) and of BTEE ( $\triangle$  and  $\blacktriangle$ ). For further details, see the text.

are tabulated in Table II. Interpretation of these data will be deferred for the discussion of this paper.

#### *Influence of pH*

Comparative measurements of the effect of pH on the peptidase and esterase activities of chymotrypsin were made with BTA and BTEE as substrates. The buffers employed were borate buffer between pH 9.2 and pH 8.2, phosphate buffer between pH 8.2 and pH 7.0, and acetate buffer at pH 6.2. Buffer concentration was 0.045 M for BTA and 0.050 M for BTEE. Substrate concentration was 0.0125 M for BTA, while

TABLE II  
*Thermodynamic Constants for Hydrolysis of BTA and BTEE by Chymotrypsin†*

Substrate	$K_m$	$\Delta H$	$\Delta F$	$\Delta S$	$k'$	$k'_0$	$\Delta E$	$\Delta H^*$	$\Delta S^*$	$\Delta F^*$	$-\frac{\Delta F}{T} + \frac{\Delta F^*}{T}$
		calories	calories	E. U.		sec. <sup>-1</sup>	calories	calories	E. U.	calories	calories
BTA	4.24 $\times 10^{-2}$	10,900	1900	+30.0	6.47 $\times 10^{-3}$	0.62‡	14,600	14,000	-13.0	17,900	16,000
BTEE	3.90 $\times 10^{-3}$	8,400	3300	+17.1	8.15 $\times 10^{-1}$	78.0‡	9,200	8,600	-21.4	15,000	11,700

† Calculated for  $T = 298^\circ$ , by means of equations (11) to (16).

$$\ddagger k'_0 = \frac{k'}{60} \times \frac{36,000}{6.25}.$$

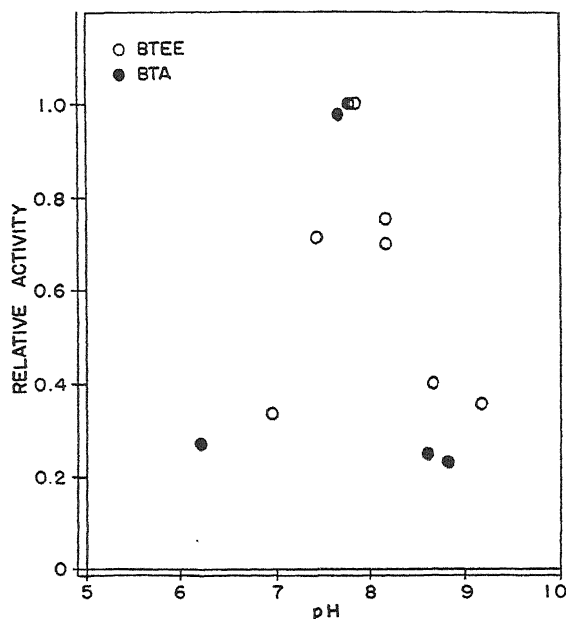


FIG. 8. The pH dependence of the hydrolysis of BTA and of BTEE by chymotrypsin. For the buffer solutions employed, see the text. ● denotes BTA, and ○ denotes BTEE. The initial substrate solutions of BTA were 0.0125 M, while the values obtained for BTEE were corrected to a common substrate concentration of 0.01 M. The apparent proteolytic coefficient determined at pH 7.8 was taken as unity and the results obtained at all other pH values were expressed as fractional values (axis of the ordinate).

measurements on BTEE were corrected from the plot shown in Fig. 4 to a common substrate concentration of 0.01 M.<sup>8</sup> The results of these

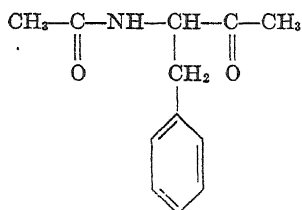
<sup>8</sup> This was necessary because of the uncertainty of the initial substrate concentra-

measurements are shown in Fig. 8 in which the apparent proteolytic coefficients for the hydrolysis of BTA and BTEE, respectively, relative to  $C = 1$  at the pH optimum, are plotted against pH. A sharp pH maximum at pH 7.8 is evident. As an approximation, the pH dependencies for the hydrolysis of the peptide and the ester are alike, suggesting that both activities are a function of the same active groups on the enzyme surface. A complete description<sup>9</sup> of the effect of pH requires determination of both  $K_m$  and  $k'$  in relation to pH.

### *Inhibitors*

It has already been shown that certain structural analogues of reaction products serve as inhibitors for a proteolytic enzyme (5). In the present work, 1-phenyl-2-acetaminobutanone-3 (PAAB) and benzoyl-L-tyrosine hydrazide (BTH) were prepared and tested as structural analogues of specific substrates. As can be seen from the structural formula, PAAB is related to acetyl-L-phenylalanine methyl ester (the analogue of the powerful substrate ATEE), the only difference being that the carbomethoxyl group,  $-\text{COOCH}_3$ , has been replaced by the methylketone group,  $-\text{COCH}_3$ . However, PAAB failed to exert any inhibitory activity whatsoever on the chymotryptic hydrolysis of BTA and BTEE when present in a substrate-inhibitor ratio of 6:1.

BTH, in which the amide group,  $-\text{CONH}_2$ , of BTA is replaced by a hydrazide group,  $-\text{CONHNH}_2$ , was likewise found to be ineffective as an inhibitor. This search for an inhibitory analogue of specific peptide and ester substrates of chymotrypsin is being continued.



PAAB (1-phenyl-2-acetaminobutanone-3)

tion in potentiometric determinations of esterase activity. Corrections to 0.01 M substrate concentration by interpolation from the curve shown in Fig. 4 may be equivocal to the extent to which the shape of this curve varies with pH.

<sup>9</sup> Such determinations have been made for the system carboxypeptidase-carboxybenzoylglycyl-L-phenylalanine (Elkins-Kaufman, E., and Neurath, H., unpublished experiments), and have shown that both  $K_m$  and  $k'$  vary with pH. Analogous measurements for the chymotryptic hydrolysis of BTA and BTEE will be reported later.

## DISCUSSION

The results of the present investigation on the substrate specificity of chymotrypsin are in accord with two of the postulates of Bergmann and coworkers (2-4): (1) Chymotrypsin catalyzes the hydrolysis of peptides containing the aromatic amino acids, tyrosine and phenylalanine. (2) Hydrolysis occurs at the peptide bond which involves the carbonyl group of these amino acids. The definition of additional structural requirements, particularly as they pertain to the  $\alpha$ -amino group of tyrosine, necessitates reconsideration of the data of Fruton and Bergmann.

Fruton and Bergmann (4) reported that L-tyrosinamide (TA) and L-phenylalaninamide (PA), the simplest conceivable substrates previously known,<sup>10</sup> are hydrolyzed at a slow rate, corresponding to apparent proteolytic coefficients of about  $C = 3 \times 10^{-4}$ . If the amino group is converted into a secondary peptide group, and an additional amino group is introduced 2 carbon atoms further removed from the  $\alpha$ -carbon atom of tyrosine, as in GTA (4), the rate of hydrolysis is increased about 30-fold ( $C = 0.01$  in 0.05 M substrate concentration). If, however, the terminal amino group is absent, as in BTA, the rate of hydrolysis is increased further, *i.e.* 20-fold with respect to GTA, and 600-fold with respect to TA, demonstrating the antagonistic influence of a positive charge on the combination between enzyme and substrate.

The data of Fruton and Bergmann on carbobenzoxyglycyl-L-tyrosinamide are in apparent contradiction to these conclusions (4). This substrate has been reported to be relatively resistant to chymotryptic hydrolysis in aqueous solution, the apparent proteolytic coefficient, calculated from a single rate value, being about  $C = 4 \times 10^{-5}$ . Since it has already been shown that carbobenzoxyglycyl-L-phenylalanine ethyl ester is a specific substrate for chymotrypsin (6), the hydrolysis of carbobenzoxyglycyl-L-tyrosinamide was reinvestigated. In 0.025 M substrate concentration and 30 per cent methanol, the apparent proteolytic coefficient was about  $C = 6.2 \times 10^{-3}$ , which is about 3 times higher than the value obtained for GTA under similar experimental conditions.

It may be concluded, therefore, that the free  $\alpha$ -amino group of tyrosyl peptides interferes with their hydrolysis by chymotrypsin, particularly when it is in close proximity to the susceptible peptide bond. Conversely, masking of this group by carbobenzoxylation, acetylation, or benzoylation increases, in the order named, the susceptibility of the resulting peptide to enzymatic hydrolysis.

The higher susceptibility of specific esters to chymotryptic hydrolysis,

<sup>10</sup> Probably the structurally simplest substrate for chymotrypsin is the methyl ester of  $\beta$ -phenyllactic acid (Snoke, J. E., unpublished experiments).

in comparison to that of specific peptides, is evidenced by the data given in Table I, and is in accord with previous comparative measurements on esterase and peptidase activities of other proteolytic enzymes (6, 7). Since the kinetic measurements have been evaluated on the basis of a two-step enzymatic reaction (equation (1)), the effect of substrate structure on both of these steps, *i.e.* formation of the stable enzyme-substrate complex and decomposition of the latter into reaction products and free enzyme, has to be considered. Since the constant,  $K_m$ , determines the equilibrium between the formation of this complex and its decomposition, comparison of  $K_m$  values for different substrates will not yield an explicit measure of the relative affinities<sup>6</sup> of the enzyme for various substrates. However, the maximum proteolytic coefficient,  $C_{max.}$ , represents the specific reaction constant per unit enzyme concentration in a range of substrate concentration sufficiently low to render this combination the rate-determining step (see equation (9)<sup>2</sup> and reference (5)). In these low ranges of substrate concentration, first order reaction kinetics apply, and comparison of  $C_{max.}$  values for various substrates will give a measure of their relative affinities. On this basis of comparison, the specific ester, BTEE, is some 8000 times more susceptible to chymotryptic hydrolysis than is the corresponding peptide, BTA, whereas the ester ATEE is only about one-fourth as susceptible as is the corresponding benzoyl derivative, BTEE.

The specific reaction constant,  $k'$ , is the rate of activation and decomposition of the enzyme-substrate complex per mg. of enzyme N per cc., in moles per liter per minute. Accordingly,  $k'_0$  is the rate<sup>7</sup> of activation and decomposition per mole per liter of enzyme-substrate complex per second (see equation (7)), and may be related to the susceptibility of the adsorbed substrate to activation and hydrolysis. According to the data given in Tables I and II, this activation and decomposition reaction proceeds about 120 times faster for the ester BTEE than for the corresponding peptide, BTA, whereas the acetylated ester, ATEE, decomposes even more readily than the benzoylated ester, BTEE. Additional interpretations are rendered available by considerations of the energetic data discussed below.

The substrate concentration dependence of apparent proteolytic coefficients varies in magnitude from substrate to substrate (Table I) and is highest for the substrate with the lowest  $K_m$  value. This is in accord with expectations, since reactions tend to approach zero order kinetics as the equilibrium between free and combined enzyme shifts to the right of equation (1) (see also equation (8)). Conversely, first order reaction kinetics predominate as the rate of combination between enzyme and substrate becomes the rate-determining step (high  $K_m$  values). The overlapping and crossing of the curves obtained when the proteolytic coefficient for

each substrate is plotted against substrate concentration (see Table I and Figs. 4 and 6) demonstrate the ambiguity which results when apparent proteolytic coefficients for the hydrolysis of two or more substrates by the same enzyme are compared at a single substrate concentration. Thus, in 30 per cent methanol and 0.025 M substrate concentration, ATEE appears to be more readily hydrolyzed than BTEE, whereas the maximum proteolytic coefficients are actually in the inverse order.

The thermodynamic data given in Table II afford an analysis of the mechanism of enzymatic hydrolysis of specific peptide and ester substrates for chymotrypsin. The equilibrium between the reactants, the stable enzyme-substrate complex  $ES$  and the activated complex  $ES^*$ , is expressed by the equilibrium constant,  $K_m$ . The formation of the stable complex is accompanied by a decrease in entropy of  $\Delta S = -30$  e.u. for the peptide (BTA), and of  $\Delta S = -17$  e.u. for the ester (BTEE). The entropy decrease may be accounted for in part by the loss of rotational motion of the substrate molecules when they combine with the enzyme. Values within the range of  $\Delta S_{\text{rot.}} = -9.3$  to  $-15.5$  e.u. have been calculated for specific peptides (15). This would account for about one-half of the total entropy loss reported in this paper. Alternatively, if it is assumed that both enzyme and substrate are hydrated, part of the entropy change may be ascribed to the "freezing out" of solvent molecules during complex formation. The higher entropy change calculated for the peptide as compared to the ester may be correlated either with a higher degree of orientation of the substrate or else with a larger number of "frozen" solvent molecules or with their higher orientation. The corresponding decrease in free energy is  $\Delta F = -1900$  calories for the peptide and  $\Delta F = -3300$  calories for the ester. The order of magnitude of these thermodynamic constants is comparable to that of those few other enzyme systems for which the temperature dependence of  $K_m$  has been determined (16).

Since  $k'_0$  is the specific reaction constant per mole of enzyme-substrate complex, the thermodynamic data given in the right-hand half of Table II are independent of the energetic changes incurred in the formation of the stable complex, and are solely characteristic of the activation process. This process is accompanied by an entropy decrease of  $\Delta S^* = -13.0$  e.u. for the peptide and of  $\Delta S^* = -21.4$  e.u. for the ester. These values are somewhat higher than those calculated by Butler (17) from data obtained under less strictly defined experimental conditions for the hydrolysis of proteins and peptides by trypsin and chymotrypsin.<sup>11</sup> The

<sup>11</sup> Since Butler's data (17) were calculated from first order reaction constants, they are not strictly comparable to the present calculations. Comparison could be made if Butler's measurements had been performed in a range of substrate concentration

entire entropy decrease observed here may be numerically accounted for by the loss of entropy of the reacting water molecules required for the hydrolysis of the peptide or ester bonds (about  $-19$  E.U. (15)).

The free energy change for the reaction,  $E + S \dots \rightarrow ES^*$  (equation (1)),  $-\Delta F + \Delta F^*$ , is approximately  $-1900 + 17,900 = 16,000$  calories for the peptide hydrolysis, and  $-3300 + 15,000 = 11,700$  calories for the ester hydrolysis.<sup>12</sup> The free energy gain resulting from the exergonic combination of enzyme and substrate compensates in both cases for only a small part of the free energy loss of the endergonic activation of the complex.<sup>13</sup>

The free energy change of activation is considerably smaller than that calculated for the acid hydrolysis of benzoylglycine and acetylglycine, *e.g.* about 30,000 calories (15). While this is in agreement with accepted theories of the mechanism of enzymatic activation, thermodynamic data for the enzymatic and non-enzymatic hydrolysis of the same substrate under otherwise identical conditions are needed to establish strict comparison, since the free energy of activation for the hydrolysis of tyrosyl peptides may differ considerably from that of N-substituted glycine. Moreover, the change in dielectric constant of the solvent by the addition of methanol undoubtedly affects the free energy of activation (18).

This work has been supported by the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service, and by the Rockefeller Foundation. Our thanks are due to Dr. Irving Klotz for valuable suggestions in the preparation of this manuscript.

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in which the first order reaction constant is independent of initial substratum concentration, in which case the calculated entropy change would be equal to  $-\Delta S + \Delta S^*$  of the present data. The values given by Butler seem to be in error since the factor  $RT/Nh = 5.7 \times 10^{12}$  and not  $5.7 \times 10^{13}$  as used by Butler. When this correction is applied to the chymotryptic hydrolysis of benzoyl-L-tyrosylglycinamide,  $\Delta S^*$  is  $-13.1$  E.U. instead of the published value of  $-17$  E.U. Similar corrections apply to all other thermodynamic constants given in that paper.

<sup>12</sup> Determinations of the thermodynamic constants of enzyme-catalyzed reactions recorded in the literature (16) have usually yielded approximated values for the overall activation reaction, the two-step nature of the reaction being neglected. The algebraic sum of  $-\Delta F + \Delta F^*$  given in the last column of Table II is the approximate value for the over-all activation reaction.

<sup>13</sup> The thermodynamic constants for the hydrolysis of carbobenzoxyglycyl-L-phenylalanine by carboxypeptidase at pH 7.5 are of the same order as those given here for the chymotryptic hydrolysis of BTA (Elkins-Kaufman, E., and Neurath, H., manuscript in preparation).



## SUMMARY

1. The substrate specificity of chymotrypsin has been reinvestigated, with particular emphasis on the rôle of a free terminal amino group of tyrosyl peptides. Benzoyl-L-tyrosinamide (BTA) has been found to be the most specific peptide substrate for chymotrypsin.

2. Specific tyrosyl esters, such as benzoyl-L-tyrosine ethyl ester (BTEE) and acetyl-L-tyrosine ethyl ester (ATEE), are hydrolyzed considerably faster than the most specific peptide substrate previously described, *e.g.* glycyl-L-tyrosinamide (GTA).

3. The specific ester and peptide substrates are hydrolyzed by chymotrypsin in the presence of methanol, the logarithm of the apparent proteolytic coefficient decreasing linearly with increasing methanol concentration up to 30 per cent.

4. The kinetics of the chymotryptic hydrolysis of GTA, BTA, BTEE, and ATEE have been evaluated on the assumption of a two-step enzymatic reaction, *i.e.* the formation of a stable enzyme-substrate complex and the subsequent activation and decomposition of the complex.  $K_m$ , the enzyme-substrate dissociation constant, and  $k'$ , the specific rate constant for the activation and decomposition reactions, have been determined for the chymotryptic hydrolysis of each of these substrates.

5. The influence of temperature on  $K_m$  and  $k'$  for the chymotryptic hydrolysis of BTA and BTEE has been determined and thermodynamic constants for the equilibrium reaction and the activation rate have been evaluated. The mechanism of enzymatic hydrolysis of these specific peptide and ester substrates has been analyzed in terms of the thermodynamic data.

6. Preliminary measurements of the pH dependence of chymotryptic hydrolysis of a specific peptide (BTA) and of a specific ester (BTEE) indicate a sharp maximum at pH 7.8, the pH-activity curves being similar for both types of substrates.

7. Preliminary, although negative, results are recorded for the inhibition of chymotrypsin by two structural analogues of specific substrates.

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# THE INFLUENCE OF FASTING AND NITROGEN DEPRIVATION ON THE CONCENTRATION OF FREE AMINO ACIDS IN RAT PLASMA\*

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(Received for publication, September 24, 1948)

The microbiological assay procedure for the determination of amino acids is particularly well suited for routine analyses of low potency materials. Its application to metabolic studies includes the measurement of "apparent free" amino acids in plasma of a number of species (1-4), "free" (5-10) and total (6-10) amino acids in urine, and free amino acids in sweat (11) and cerebrospinal fluid (12). The use of these methods for such materials which may contain amino acids in combined forms, without preliminary hydrolysis, has been criticized. Some peptides can replace one or more of their constituent amino acids for lactic acid bacteria usually employed in assays (13-15). Nevertheless, such methods seem useful for studies in which comparative results are desired. They might conceivably become a useful tool for laboratory detection of pathological conditions.

Schweigert *et al.* (2) and Dunn *et al.* (1) have employed these procedures for the determination of the tryptophan content of rat plasma and of tungstic acid filtrates of human blood fractions, respectively. Hier and Bergeim (3, 4), applying these procedures to tungstic acid filtrates, have determined the normal fasting plasma values for ten amino acids for man and the dog. Hier (16) studied the effect of administration of large doses of a number of single amino acids on the plasma levels of twelve amino acids. These workers (3) showed that acceptable recovery values were obtained, that the values were reproducible, and that the results were not affected by nembutal anesthesia, by prolonged contact of the protein-free plasma with the precipitate, or by long storage of the filtrate at low temperature.

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Nutrition Foundation, Inc., New York, and by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

We wish to acknowledge the assistance of Miss Tara Deodhar in the early phases of this study.

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In the studies reported here the microbiological assay method has been employed in the determination of free<sup>1</sup> amino acids in the plasma of the rat under various dietary conditions.

#### EXPERIMENTAL

*Care of Animals*—Sprague-Dawley, male rats from 200 to 300 gm. in weight were used. They were fed a stock diet for 2 weeks and then shifted to the purified ration at least 1 week prior to the beginning of the dietary studies. A typical purified ration of the following composition was used: sucrose 71, crude casein 18, Salts IV (17) 4, corn oil 5, and a dry vitamin mixture<sup>2</sup> 2.

1 week before the blood samples were to be drawn the rats were selected to give similar weight distribution in each experimental group. In the first experiment ten rats were used for each group; in three later experiments only 3 rats were used per group. This reduction in number was made possible by the development of the micro technique (18). Eleven groups were used in each experiment. One served as control, receiving the ration *ad libitum* until the blood sample was drawn. The remaining groups were deprived of the entire ration or of the protein of the ration at various periods during the experimental period. The protein-free ration differed from the one described above only in that the casein was replaced by an equal quantity of sucrose. At the time blood was drawn, rats had been fasted or depleted of protein for 1, 2, 3, 5, or 7 days. In later experiments, additional groups were fed the protein-free diet for 14 and 21 days.

Since marked changes in the concentrations of some amino acids occurred as a result of 1 day of fasting, additional animals were used to determine the effect of short periods of fasting and the range of values for rats under given dietary conditions.

In one experiment five animals of similar size and of the same age and dietary history were used to determine the approximate individual variation in plasma values of non-fasted rats. The samples were treated in the same manner and were analyzed simultaneously. In the course of three other experiments, carried out over a period of more than a year, the effect of 6 to 9 hours of fasting was studied.

*Drawing Blood and Preparation of Plasma*—The rats were anesthetized with approximately 10 mg. of sodium amytal per 100 gm. of body weight

<sup>1</sup> "Free" as used throughout implies microbiologically available amino acids and, as pointed out in the text, it may include combined forms as well as free amino acids.

<sup>2</sup> Each 2 gm. portion of the vitamin mixture contained 0.2 mg. of thiamine hydrochloride, 0.3 mg. of riboflavin, 0.25 mg. of pyridoxine, 2 mg. of calcium *D*-pantothenate, 1.5 mg. of niacin, 100 mg. of choline chloride, 0.01 mg. of biotin, 0.02 mg. of pteroylglutamic acid, and 10 mg. of inositol. 2 drops of halibut liver oil were administered each week by dropper.

administered intraperitoneally in one dose. Blood was drawn by cardiac puncture with a No. 23 needle. Heparin was employed as the anticoagulant at a concentration of 0.1 to 0.2 mg. per ml. of blood. The blood from the rats in each group was pooled and then centrifuged for 20 minutes. The plasma was measured into 50 ml. glass-stoppered centrifuge tubes and the proteins were precipitated with sodium tungstate and sulfuric acid as described by Hier and Bergeim (3). The precipitate was removed by centrifugation. The protein-free plasma was neutralized to pH 7.0 with  $N$  NaOH and stored in a stoppered test-tube or flask preserved at 5° or in a frozen state under toluene until analyzed.

*Assay Procedure*—The filtrate, representing a 3-fold dilution of the plasma, was measured into 10 × 75 mm. tubes in volumes from 0.02 to 0.1 ml. and the microbiologically available amino acids were determined as previously described (18). In the first experiment 2 ml. volumes were used (19). The Cannon dispenser and titrator<sup>3</sup> was employed throughout. Glutamic acid could not be determined under these conditions because of the partial conversion of biologically active glutamine present in plasma to the inactive pyrrolidonecarboxylic acid during the sterilization of the assay tubes (20). Aspartic acid was determined in some plasma samples, but the small quantity of free aspartic acid present (trace to 8  $\gamma$  per ml.) made the assay unreliable. Good agreement was found at the various assay levels over the entire range of the standard curves for all of the amino acids determined. In later experiments glycine determinations were included to make a total of thirteen amino acids exclusive of glutamic acid and aspartic acid.

Recovery of amino acids added to the plasma before precipitation were 90 to 110 per cent in most cases. Limited experiments indicated that the anesthetic had little, if any, effect on the plasma amino acid levels.

#### RESULTS AND DISCUSSION

The variation found in the amino acid content of plasma from five rats sampled at the same time is shown in the second column of Table I. The average deviation is approximately  $\pm 10$  per cent for all amino acids. This variation is approximately twice that encountered in repeated assays of the same plasma; therefore, a large part of the variation is a result of true differences between individual rats. These animals were not fasted, hence they might have been in different stages of protein assimilation. In other experiments of a similar nature, variations comparable to those shown here were found for a few amino acids on rats fasted for 6 to 8 hours.

When pooled samples of blood from three animals per group are analyzed

<sup>3</sup> Cannon, M. D., in press.

simultaneously, a variation of more than 10 per cent from the control group is therefore considered to be significant.

The variation in results obtained with single determinations (six tubes in each) of amino acids in the plasma of individual animals in three different experiments (the third column, Table I) was approximately double that just described. The results emphasize the importance of adequate control groups in every experiment and the use of pooled samples from several animals when it is not practicable to analyze samples from each of the individual animals. It is interesting that the values for most of the amino acids in rat plasma agree with those reported for the human being

TABLE I  
*Amino Acid Content of Plasma from Adult Rats*

Amino acid	Not fasted, 5 rats on same day	Fasted 6 to 9 hrs., 9 to 14 animals in 3 experiments
Leucine .....	26.6 $\pm$ 2.3*	20.7 $\pm$ 4.3*
Phenylalanine .....	13.7 $\pm$ 1.1	11.0 $\pm$ 1.5
Tryptophan .....	16.8 $\pm$ 1.2	14.5 $\pm$ 2.5
Valine .....	26.7 $\pm$ 2.0	25.6 $\pm$ 5.2
Histidine .....	9.7 $\pm$ 0.6	12.2 $\pm$ 1.3
Lysine .....	58 $\pm$ 7	72 $\pm$ 13
Isoleucine .....	13.2 $\pm$ 1.8	13.5 $\pm$ 2.6
Glycine .....	22.7 $\pm$ 2.1	30.6 $\pm$ 6.3
Proline .....	43 $\pm$ 4	36 $\pm$ 14
Tyrosine .....	22.3 $\pm$ 2.5	14.7 $\pm$ 2.9
Methionine .....	9.5 $\pm$ 0.7	9.5 $\pm$ 1.3
Threonine .....	44 $\pm$ 5	42 $\pm$ 8
Arginine .....	32.1 $\pm$ 4.3	38.5 $\pm$ 4.4

\* Mean deviation from the mean.

and the dog (4). The lysine, arginine, and threonine concentrations for the rat plasma were higher than for the other two species.

In the experiments discussed below each dietary variation was imposed on three animals or more. Equal volumes of blood from each animal were mixed and the resulting pooled sample analyzed simultaneously with those from each of the other experimental groups. The fasting and nitrogen deprivation experiments were repeated four times. Each time the major trends appeared, but changes in certain amino acids resulting from these dietary variations were not always of the same magnitude. The results of these four experiments have been averaged and are shown graphically in Fig. 1.

The changes in the concentration of some amino acids were rather marked, while others remained quite constant during periods of acute

negative nitrogen balance resulting from deprivation of protein or the entire ration. Similar patterns were noted for leucine, tryptophan, valine, isoleucine, methionine, and to a slight degree phenylalanine. In each case the total fasting resulted in little change in plasma levels, although in several instances there was an increase after 1 day. On the other hand, a marked and sometimes rapid decline resulted when protein was removed

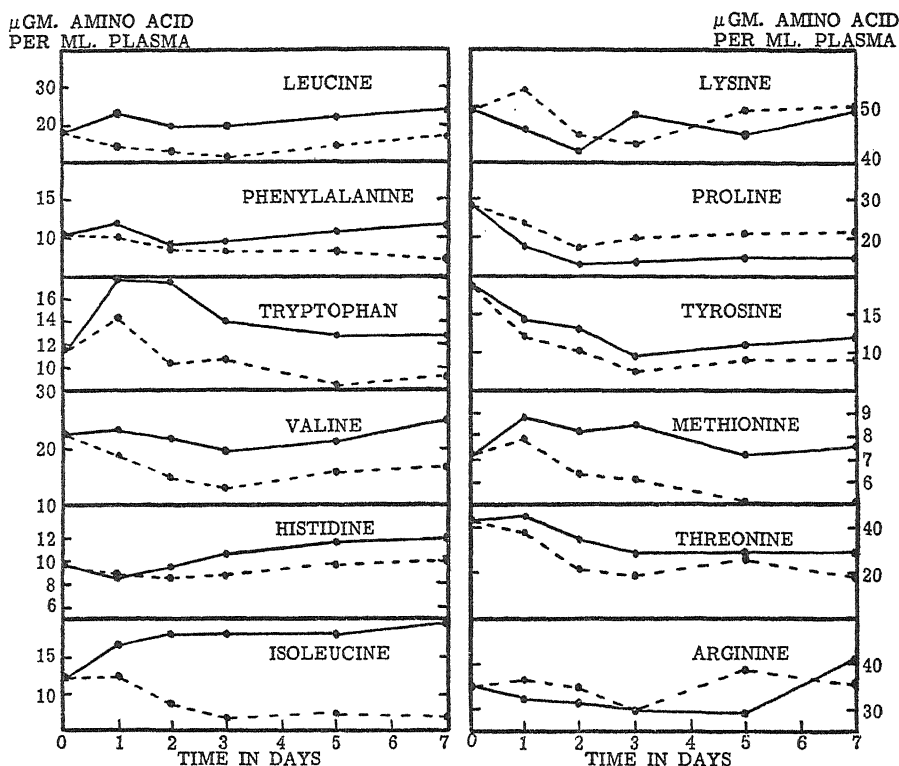


FIG. 1. The effect of fasting and nitrogen deprivation on the concentration of free amino acids in rat plasma. The dash line represents the results from animals on nitrogen deprivation; the solid line, results from animals on total fasting.

from the ration. This decline ceased after 3 to 5 days in most cases. In one experiment in which the nitrogen-free diet was fed for 3 weeks, the results indicated little change in values after 1 week.

Other amino acid concentrations showed various trends. The histidine content of plasma decreased slightly for 1 to 2 days and then returned to normal or slightly above. Threonine, proline, and tyrosine concentrations declined markedly during the first 2 to 3 days and remained low in both the

fasted and protein-depleted animals. The lysine content of plasma was not affected by deprivation, while arginine varied between rather narrow limits.

The relatively small changes in hematocrit values noted in two experiments indicate that the differences in plasma concentrations of amino acids are not the result of altered water content. In the groups receiving the nitrogen-free ration, a gradual increase in hematocrits from 40 to 41 to 43 to 45 per cent was noted as protein depletion progressed. Fasting resulted in a gradual hemoconcentration with hematocrit values of 49 to 50 per cent on the 7th day. This might account, in part, for the failure of the fasted animals to show the decrease in the levels of some amino acids noted in the protein-depleted rats. Losses of body weight during the 7 day experimental period averaged 31 per cent for the fasted rats and 13 per cent for those receiving the nitrogen-free diet. In preliminary experiments some animals died on the 7th day of fasting. Similar losses in weight, but lower survival, were reported by Roche (21) for rats under conditions similar to those reported here.

Since the most rapid changes in the plasma amino acid concentrations occurred during the first 24 hours of fasting, an additional experiment was performed to determine the effect of fasting for periods of 3, 6, 9, 12, and 24 hours. The changes which occurred from 6 to 9 hours were of particular interest, since that period has been used routinely to obtain rats in a post-absorptive state. It should be pointed out that the rats used in the fasted and protein-depletion studies just described were not fasted prior to the removal of blood samples.

The changes in plasma amino acid content resulting from fasting are shown graphically in Fig. 2. Unfortunately a control group receiving food until the time blood was removed was not included. There was no general pattern of change for any of the amino acids during the 3 to 24 hour period. The concentrations of most amino acids increased as the fasting progressed, with maximum values at 9 to 12 hours and a decline thereafter to values approaching or going well below those for the animals fasted 3 hours. For leucine, lysine, proline, tyrosine, arginine, and glycine the values varied more than others on a percentage basis. These results indicate that care should be exercised in such studies to standardize the duration of the fasting period used to get a postabsorptive state.

Christensen and Lynch (22, 23) have presented data which indicate that the conjugated  $\alpha$ -amino nitrogen in tungstic acid filtrates of the plasma of normal human beings is not diffusible through cellophane and has some properties of proteins. Tungstic acid filtrates contained less conjugated  $\alpha$ -amino nitrogen than those from picric and trichloroacetic acid. It was reproducible, but quite variable, in different individuals and was unaffected



by storage of whole plasma. Proteolysis did not occur when the filtrate stood in contact with the precipitate.

The activity of this protein-like fraction for microorganisms has not been determined, but since it is non-dialyzable, it probably is not available to lactic acid bacteria.

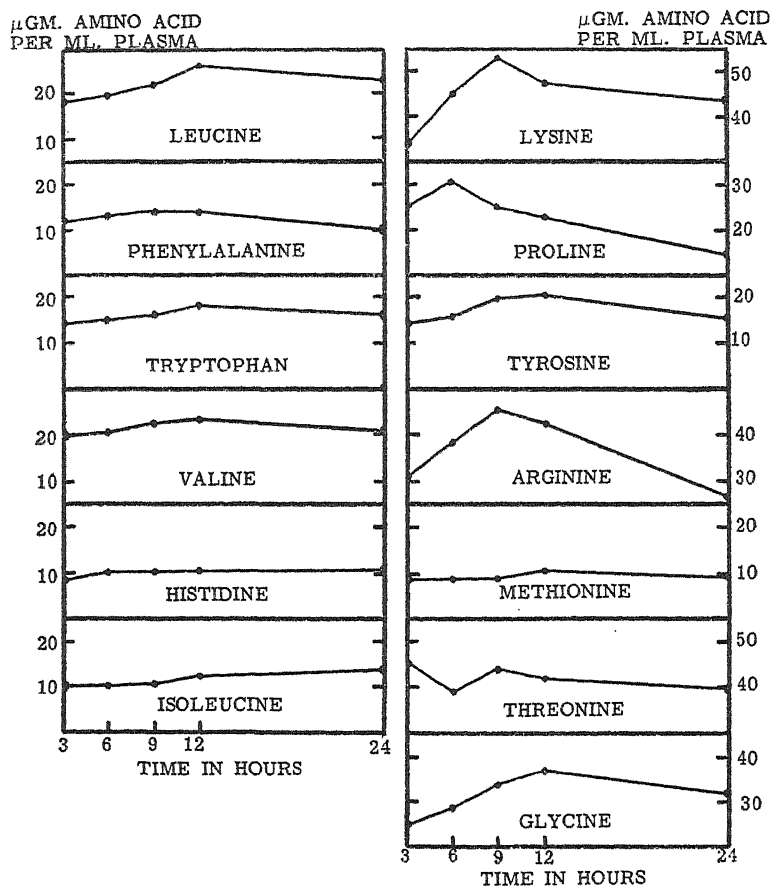


FIG. 2. The effect of short periods of fasting on the concentration of free amino acids in rat plasma.

Since there appears to be little, if any, diffusible, conjugated forms of amino acids in tungstic acid filtrates, the procedure used here for the preparation of protein-free filtrates of plasma for microbiological assay of free amino acids appears to be the most suitable thus far proposed. Hier and Bergeim (3) found that such filtrates were very stable to prolonged contact

with the precipitate and to storage at low temperatures. This has been confirmed in these studies. The increase in microbiologically available amino acids of tungstic acid filtrates after acid hydrolysis was 25 to 400 per cent, with most amino acids increasing approximately 200 per cent.

The values given in Table I for fasted animals correspond to approximately 2.9 mg. per cent (3.6 with glycine) of  $\alpha$ -amino nitrogen, assuming that only free amino acids were measured by the methods employed. The normal postabsorptive value for amino acid nitrogen of plasma is 6.4 mg. for rats (24). This means that approximately half of the amino acid nitrogen of rat plasma is accounted for by the twelve amino acids measured. Further, if we assume that the rat, like the human being, has 18 to 25 per cent of the ninhydrin nitrogen present as glutamine (25) and 24 per cent as glycine and alanine (26), the values presented here would account for 90 to 100 per cent of the total amino acid nitrogen, and serine, hydroxyproline, cystine, and any free glutamic acid would not be included.

The general finding that the concentration of amino nitrogen in plasma (27) is not greatly changed under conditions of metabolic stress resulting from fasting has been confirmed in these studies.

Individual amino acids vary considerably more, but since a change in concentration of one is accompanied by a compensatory change in another amino acid, the methods which measure the amino acids collectively have detected only gross changes involving simultaneous changes in the same direction.

The values reported here for fasted and protein-depleted rats will serve as a base from which the effect of other dietary changes involving inanition and negative nitrogen balance can be measured. It is realized that the values may not be absolute, since small amounts of peptides might be active for the test organism. For exploratory types of experiments in which comparative data will suffice, they may be of considerable value. Application of these methods which permit a nearly complete amino acid analysis of plasma from 4 to 6 ml. of blood to the study of pathological conditions in man and of protein metabolism in small animals seems possible. Such quantitative studies of the concentration of the "free" amino acids in the blood and other tissues will lead to a better understanding of protein assimilation and synthesis in the animal body.

#### SUMMARY

1. A micro adaptation of the microbiological assay procedure has been used to measure the concentrations of thirteen amino acids in tungstic acid filtrates of rat plasma.
2. The variations in normal values for individual animals receiving an

18 per cent casein ration *ad libitum* and after brief fasting have been determined.

3. The effects of fasting for periods up to 24 hours and of fasting or nitrogen depletion for 7 days have also been studied by employing pooled samples of plasma from three rats or more. No general response pattern was noted for all amino acids, though a number of them behave similarly. The concentration of a number of amino acids increased in the early stages of fasting and then returned to normal as the fasting progressed. Nitrogen depletion caused a decline in the plasma concentrations of most amino acids, in some cases to one-half of normal values. The levels of other amino acids were changed very little as a result of deprivation.

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# AMINO NITROGEN RETENTION IN PLASMA FOLLOWING TRAUMA\*

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(Received for publication, October 8, 1948)

The disorder of protein metabolism leading to excessive loss of nitrogen following moderate to severe injury has been studied extensively. However, with the exception of reports on amino nitrogen metabolism in hemorrhagic shock, the literature is poor in work on the fate of amino acids in the body after trauma. Conceivably, in addition to posttraumatic "excessive protein catabolism" (1) an associated defect in the utilization of amino acids might also occur after injury. Such a circumstance could account for the apparent inefficacy of amino acid mixtures administered parenterally in the restoration of nitrogen equilibrium in various clinical conditions (2-5). Accordingly, observations were made of the rate of decrease of  $\alpha$ -amino nitrogen in the plasma following injection of L-lysine monohydrochloride in dogs, before and after operation (laparotomy). Lysine was selected for this study because of its restricted metabolic rôle (6-8), which seems to be limited to the elaboration of body protein.

## EXPERIMENTAL

*Procedure*—Three healthy adult mongrel dogs, 20 to 30 kilos in body weight, were used. They were maintained exclusively on Purina laboratory chow during experimental periods, with a supplement of horse meat during rest periods. The dogs were kept in metabolism cages in an air-conditioned room for at least 1 week prior to the experiment and then throughout control and experimental periods.

Each animal served as its own control. During the control period several amino acid loading curves were determined on each animal as follows: After an 18 hour fast, a blood specimen was drawn. This was followed by the rapid injection into the saphenous vein of approximately 2.9 mg. of  $\alpha$ -amino nitrogen per kilo of body weight as L-lysine monohydrochloride. The appropriate dose of the amino acid was dissolved in 10 ml. of 0.9 per cent sodium chloride solution. At exactly 5 and 10 minutes after injection two

\* The Bureau of Medicine and Surgery of the Navy does not necessarily endorse the views or opinions which are expressed in this paper.

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additional blood samples were taken from the radial veins. Heparin was employed throughout as an anticoagulant.

Laparotomies were performed under sodium pentobarbital anesthesia following the administration of morphine sulfate (0.004 gm. per kilo) and atropine sulfate (0.001 gm.), doses somewhat less than those recommended by Seevers (9). The peritoneum was opened through a 5 to 6 inch paramedian incision, and after gentle palpation of the abdominal viscera, careful anatomical closure of the incision was effected. Usual aseptic technique was employed, the entire procedure taking about 40 minutes.

48 hours after laparotomy amino acid loading determinations were made and repeated every 2 or 3 days until control values were obtained. After a rest period of 4 weeks the effect on plasma amino acid retention of surgical anesthesia and premedication without trauma was tested on the same dogs in a similar manner.

*Methods*—Plasma amino nitrogen levels were determined by the ninhydrin method of Hamilton and Van Slyke (10, 11). Blood urea nitrogen was determined in tungstic acid filtrates by a steam distillation method in which a modification of Redemann's apparatus (12) was employed. The copper sulfate specific gravity technique of Phillips *et al.* was used for the estimation of plasma protein concentrations (13).

In separate experiments liver function was tested by the bromosulfalein method before and 48 hours after laparotomy. 5 mg. per kilo of body weight of the dye were injected and one blood sample was taken at 30 minutes. Color comparisons were made according to Drill and Ivy (14), who employed the original standards of Rosenthal and White.

### Results

No postoperative complications were noted. All incisions healed by first intention without evidence of infection. The animals were apparently normal 24 hours after operation.

The rate of decrease of  $\alpha$ -amino nitrogen in the plasma following parenteral injection of L-lysine monohydrochloride is shown in Table I. It indicates that during the control or preoperative periods there was a consistent fall in amino nitrogen values between 5 and 10 minutes following injection. The 5 minute maximum peak has been repeatedly demonstrated in normal animals with DL-alanine as well as with L-lysine in preliminary experiments in which comparable doses were administered.

Following laparotomy, in contrast to the results for controls, the 10 minute values were consistently elevated above the 5 minute level. This retention of plasma amino nitrogen was observed in all amino acid curves made during a period of approximately 1 week, following which there was a gradual return to normal.

TABLE I

*Effect of Laparotomy on Plasma Amino Nitrogen Retention Following Parenteral Injection of 2.9 Mg. per Kilo of  $\alpha$ -Amino Nitrogen (L-Lysine Monohydrochloride)*

The values are corrected for blood urea nitrogen.

Dog No.	Pre- or postoperative day	Body weight	Plasma sp. gr.	$\alpha$ -Amino nitrogen, mg. per 100 ml.		
				0 min.	5 min.	10 min.
		kg.				
9 ♀	7	21.8	1.027	4.86	5.70	5.50
	6	21.8	1.027	4.89	5.85	5.36
	2	22.0	1.027	4.91	6.00	5.49
9. Laparotomy ♀	2	21.0	1.027	5.51	6.09	6.19
	3	21.2	1.026	5.17	5.87	6.04
	6	21.0	1.027	4.60	5.55	5.72
	9	21.3	1.027	4.84	5.82	5.84
	12	20.9	1.027	4.93	5.98	5.78
	15	21.0	1.027	5.02	5.98	5.56
	18	21.2	1.027	5.02	5.69	5.58
	25	22.4	1.027	4.81	5.54	5.32
6 ♂	32	19.7	1.023	5.03	5.95	5.69
	30	19.8	1.023	4.52	5.78	5.19
	23	19.0	1.024	4.35	5.40	4.76
	7	19.1	1.024	4.59	5.89	5.05
6. Laparotomy ♂	2	18.2	1.023	4.67	5.69	5.87
	3	18.2	1.023	4.64	5.59	5.77
	5	18.0	1.024	4.62	5.52	5.72
	7	18.6	1.023	4.68	5.57	5.00
	10	18.0	1.024	4.72	5.52	4.90
	18	18.5	1.023	4.63	5.41	4.95
10 ♂	32	29.1	1.027	4.82	5.62	5.41
	30	28.2	1.026	4.75	5.47	5.24
	23	28.5	1.027	4.80	5.47	5.00
	7	29.0	1.028	4.69	5.45	5.13
10. Laparotomy ♂	2	27.5	1.027	4.71	5.33	5.56
	3	28.0	1.026	4.71	5.65	5.87
	5	28.0	1.027	4.74	5.59	5.79
	7	28.3	1.027	4.88	5.75	5.61
	10	28.0	1.027	4.58	5.51	5.32
	14	27.8	1.027	4.57	5.56	5.18
	18	28.4	1.026	4.51	5.47	4.92

The total plasma protein concentration as determined by the specific gravity method was quite constant throughout both the control and ex-

perimental periods. In each animal, however, a small but definite loss in weight occurred after operation.

The surgical procedure seemed to have no definite effect on the fasting amino nitrogen level of the plasma. In Dog 9 the early fasting levels were somewhat higher after laparotomy, a similar tendency being suggested by the values obtained for Dog 6. The third animal (Dog 10) showed no significant change.

Table II, containing the data of observations made before and 48 hours following anesthesia, shows that in no case was the 10 minute amino nitrogen level of the plasma elevated in excess of the 5 minute value, as was the case following operation. The decrease, however, between the 5 and 10 minute specimens after anesthesia was not as great as that found in the

TABLE II

*Effect of Anesthesia and Premedication on Plasma Amino Nitrogen Retention Following Injection of 2.9 Mg. per Kilo of  $\alpha$ -Amino Nitrogen (L-Lysine Monohydrochloride)*

The values are corrected for blood urea nitrogen.

	Time	$\alpha$ -Amino nitrogen, mg. per 100 ml.		
		Dog 6	Dog 9	Dog 10
	<i>min.</i>			
Preanesthesia	0	4.58	4.76	4.74
	5	5.60	5.72	5.53
	10	5.05	5.16	5.28
Postanesthesia	0	4.50	4.65	4.57
	5	5.38	5.47	5.30
	10	5.24	5.19	5.12

controls. These differences are small and of questionable significance. Anesthesia had no effect on weight or on the specific gravity of the plasma.

The results of the pre- and postoperative bromosulfalein tests consistently showed a retention of 5 per cent or less of the dye at 30 minutes in the three animals used. The postoperative determinations were made 48 hours after laparotomy.

#### *Comment*

The data presented are interpreted as indicating a possible defect in amino nitrogen metabolism in dogs subjected to laparotomy. Although the increment in amino nitrogen retention in plasma after operation is not large in any one instance, the consistency of the tendency is indicative of such a possibility. Apparently, after surgery the dog is unable to metabolize amino nitrogen (L-lysine monohydrochloride) as well as before operation. This finding suggests an additional aspect of the deranged me-



tabolism consequent to injury. The surgical procedure itself rather than anesthesia seems to be responsible for the defect observed.

The question of hepatic insufficiency arises quite naturally in this connection. In the present study the bromosulfalein test has been employed in an attempt to estimate any coincident liver damage. Although obviously the absolute competence of the liver in its varied physiological activities is not measured by this test, in the detection of early liver damage in dogs it appears to be somewhat more sensitive than other clinical methods (14). By its use, no evidence of impaired liver function was obtained 48 hours after operation, the time at which the suggested defect in amino nitrogen utilization became apparent.

Dogs rendered hypoproteinemic by maintenance on a protein-deficient diet exhibit plasma amino acid retention upon administration of casein hydrolysate (15, 16). Goettsch *et al.* consider this phenomenon a consequence of liver insufficiency resulting from parenchymal damage of the type described by Elman and Heifetz (17). On the basis of constant and relatively high plasma protein levels as well as the failure to detect impaired liver function by the method employed, such an explanation of our results seems rather unlikely.

Man *et al.* (18) have reported that a depression of plasma amino nitrogen occurs in humans after surgical procedures. This effect is not shown by our data for dogs, and may possibly be accounted for by a species difference in reaction to injury. However, laparotomy by itself is less traumatic than many of the procedures performed on the patients studied by these authors, and in our animals the insignificant blood loss at operation, absence of infection, and the fact that fluids were not given parenterally could all have been factors permitting the higher postoperative levels of plasma amino nitrogen observed. An early transient depression could have escaped detection, as our first determinations were not made until 48 hours after operation.

At the present time an adequate explanation of our results is not possible. In the absence of conclusive evidence of liver impairment, the findings are interpreted as possibly representing a defect, the result of injury, in the normally rapid tissue fixation (19, 20) of amino nitrogen (L-lysine).

#### SUMMARY

Amino nitrogen retention in plasma occurs in dogs subjected to laparotomy following parenteral administration of L-lysine monohydrochloride.

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## BACTERIMETRIC STUDIES

### II. THE RÔLE OF LYSINE IN BACTERIAL MAINTENANCE\*

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(Received for publication, July 9, 1948)

In connection with studies concerning the patterns of the growth responses of *Streptococcus faecalis* (ATCC No. 9790) to individual amino acids, it was found (1) that, when lysine is the growth-limiting nutrient, initial growth is followed by a decrease of bacterial density which in velocity approaches that of the increase in bacterial density during the logarithmic phase of growth. Under our experimental conditions, this phenomenon is not observed when growth is limited by either arginine, histidine, isoleucine, leucine, methionine, threonine, or valine. The present paper is devoted to the description and discussion of some aspects of the lysine phenomenon.

The composition of the medium and methods of culture and measurement have been described (2). Fig. 1 shows an experiment illustrating the nature of the process under study. By conducting it at 29° instead of at 38° the velocity of the bacterial changes could be reduced to conveniently measurable rates. At the time of the initial reading, bacterial density tends to be linearly proportional to the amount of lysine present in the medium, up to the 250  $\gamma$  level. However, with quantities of 200  $\gamma$  of lysine or less, the bacteria formed undergo a rapid lysis, while at 250  $\gamma$  and above they appear increasingly stable. The interpretation seems obvious that at levels of 200  $\gamma$  and below the medium becomes rapidly depleted of lysine and maintenance of the bacteria depends on the presence of excess lysine in their environment.

Bacterimetric determinations of lysine, with different media and different organisms, have been described by several authors and a process of rapid lysis has apparently not been encountered. Having convinced ourselves that it does not occur in the medium of Stokes *et al.* (3), we attempted to determine which of the several ways in which our medium differs from the former are responsible for the instability of lysine-limited growth. The experiment summarized in Table I, in which the medium of Stokes was supplemented in seven additive steps by the extra components of our medium, without, however, withdrawing those which in our regular

\* Aided by a grant from Mary Curtis Zimbalist. Reported in part before the American Society of Biological Chemists, Atlantic City, March, 1948 (1).

medium are omitted, shows that the only change which gives rise to lysis is the increase of the phosphate concentration from 0.005 to 0.30 M.

The next objective was to determine whether the lytic process is the result of the high ionic strength of the phosphate buffer, or of the pH being stabilized near 6.5, or whether phosphate itself is specifically responsible. The experiment described by Fig. 2 showed that neither sodium chloride

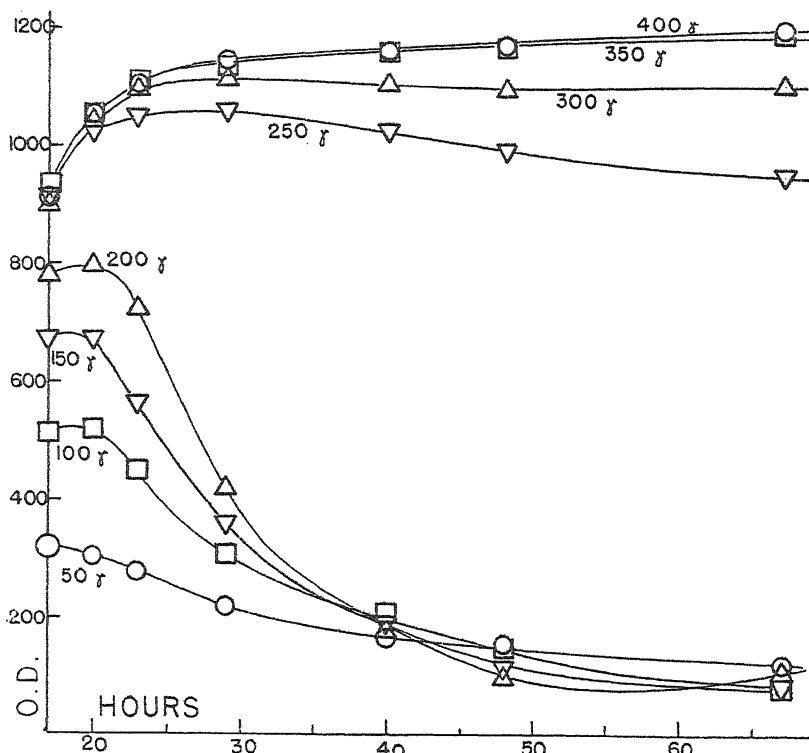


FIG. 1. Growth response to supplements of L-lysine (each level in duplicate) in the provisional buffered medium. The abscissa shows hours of incubation at 29°, the ordinate gross optical densities (2), uncorrected for color or deviation from Beer's law.

of the same ionic strength as the phosphate buffer nor a citrate buffer of the same pH and the same buffering capacity leads to lysis. Apparently, therefore, the phenomenon involves a specific effect of phosphate ion. The other effects noted, retardation of growth by citrate and increase of apparent bacterial density by sodium chloride, remain to be further investigated.

The high phosphate concentration having been established as the crucial

TABLE I  
Composition of Medium and Stability of Lysine-Limited Growth

Composition of medium*	Net optical density after 16 hrs. incubation	Change in net optical density after 46 hrs. incubation
		<i>per cent</i>
A. Ingredients according to Stokes (3)	465	-10
B. A, + 50 $\gamma$ each of asparagine and glutamine	481	-9
C. B + hydrolysate of 2 mg. yeast nucleic acid and 0.2 mg. thymine	388	-9
D. C + 0.05 $\gamma$ biotin	524	-2
E. D + 10 $\gamma$ nicotinamide	535	-1
F. E + 100 mg. glucose	469	0
G. F + 6 mg. ammonium sulfate	456	-3
H. G + 420 mg. sodium phosphate buffer, pH 6.5	271	-95

\* The medium is limited by 125  $\gamma$  of lysine per tube. Three inoculated tubes and one non-inoculated tube of each composition were incubated at 37-38°.

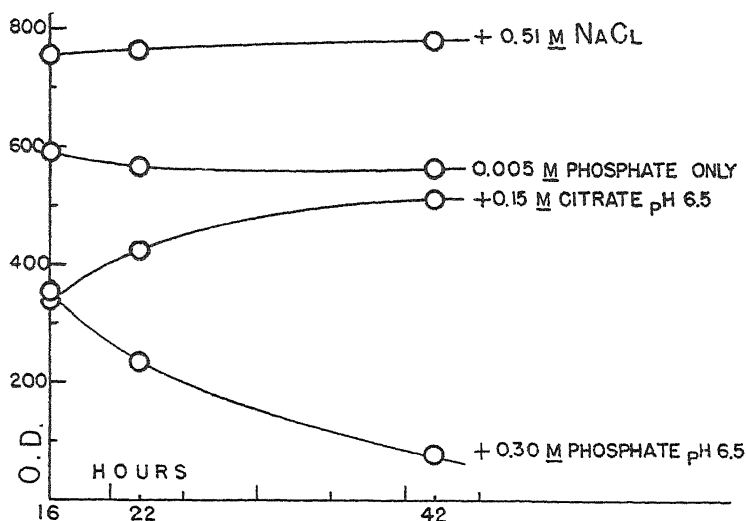


FIG. 2. The rôle of different factors in the lysis of bacteria grown in a medium limited by lysine (150  $\gamma$  per 10 cc). The provisional medium, with omission of 0.3 M sodium phosphate, was used (two tubes each) alone (0.005 M phosphate) and with additions of 0.5 M NaCl, 0.15 M sodium citrate, or 0.3 M sodium phosphate; 37.7°.

factor of the medium, the possibility was considered experimentally that the lysine-free high phosphate medium possesses the capacity to disintegrate bacteria, either *per se* or by virtue of metabolites it may contain as a result of bacterial growth. For this purpose tubes were prepared containing no

added lysine or 150  $\gamma$  of lysine, with either the low phosphate or the high phosphate medium. They were inoculated and incubated at 29°. After approximately 20 hours, when optical density readings indicated that the phase of decline had just begun in the tubes containing 150  $\gamma$  of lysine in the high phosphate medium, the tubes were centrifuged, the medium was

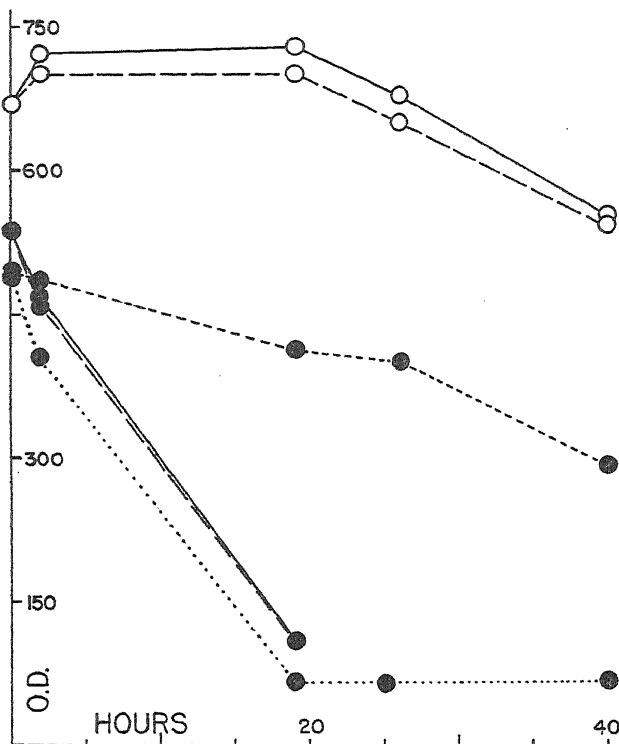


FIG. 3. Relations between the nature of the medium and lysis. The circles identify the origin of the bacteria and the lines indicate the nature of the medium in which they were suspended after their initial growth. ●, cells grown in a 150  $\gamma$  lysine, high phosphate medium; ○, cells grown in a 150  $\gamma$  lysine, low phosphate medium. The solid line indicates the 150  $\gamma$  lysine, high phosphate medium after bacterial growth; the dash line, the 0  $\gamma$  lysine, high phosphate medium after growth; the curve with short dashes, the 150  $\gamma$  lysine, low phosphate medium after growth; the dotted line, the 0  $\gamma$  lysine, low phosphate medium after growth.

decanted, and the bacteria were resuspended in a new medium. Thus cells grown in the presence of 150  $\gamma$  of lysine in the low phosphate medium were resuspended in the supernatant liquid from a culture grown in the presence of 150  $\gamma$  of lysine in the high phosphate medium, or in the supernatant of a blank culture (initially containing only traces of lysine) in high phosphate

medium. Likewise, cells grown in the high phosphate medium containing 150  $\gamma$  of lysine were resuspended in low phosphate supernatants from either a 150  $\gamma$  lysine culture or a blank culture. As controls, cells grown in high phosphate medium were resuspended in high phosphate supernatants of a culture of their own type (initial lysine content 150  $\gamma$ ) or of a blank culture. The new suspensions resulting from these exchanges were again incubated at 29°. The resulting effects, in terms of optical density readings, are shown in Fig. 3. It is evident that cells grown in a lysine-limited medium of high phosphate content undergo lysis at essentially the same rate, whether they are suspended in a high phosphate or a low phosphate medium. The reduced rate of decline in the 150  $\gamma$ , low phosphate medium is presumably attributable to the counterbalancing effect of some new growth resulting from residual lysine contained in this medium. The fact that, by contrast, the pattern of decline does not differ in the high phosphate medium between the supernatant from a 150  $\gamma$  culture and a blank culture indicates that this type of medium is, at the time of beginning lysis, substantially lysine-free. The two upper curves in Fig. 3 show the pattern resulting when cells grown in the low phosphate medium are suspended in high phosphate media. The small initial rise in optical density may represent additional growth or merely reflect a change in the optical properties of the bacteria associated with the change in the osmotic pressure of the medium. The slow decline setting in after 20 hours levels off on the 5th day of incubation at optical densities of 300 to 400 and in this respect (as well as in velocity) differs from the lysis of cells originally grown in the high phosphate medium, which proceeds practically to completion. The fact that the partial lysis observed in the high phosphate culture of cells coming from a low phosphate medium is essentially the same, regardless of the initial lysine content of the resuspension medium, suggests that a partial transformation of resistant to lysing cells may be involved, rather than lysis of cells newly formed from residual lysine present in the new medium.

The preceding evidence seems to justify the conclusion that lysis issues from the cell itself, and is not inflicted upon the cell by the medium. It should be mentioned that in parallel experiments, omitted in order to simplify the graph, the centrifuged bacteria of the original cultures were washed three times with saline (0.14 M NaCl) by suspension and centrifugation, without significant changes in the results of the secondary incubations. One must conclude, then, that lysis occurs as an expression of properties which cells acquire while growing in a high phosphate, low lysine medium. If the growth medium has a low phosphate or a high lysine concentration, or both, the resulting cells are not subject to immediate lysis, even in the type of medium which has given rise to lysis-susceptible cells.

The data plotted in Fig. 1 yield some information on the kinetics of the

lytic process. Table II shows selected data of Fig. 1, expressed in relative bacterial density units, and derived values for the rate of disappearance of bacteria. These results are expressed in terms of monomolecular kinetics as the half time of lysis; *i.e.*, the time required for halving the bacterial density. The data indicate, especially for the two higher lysine levels, reasonable compliance with the monomolecular law. At the lower lysine levels, lysis proceeds more slowly and the monomolecular constant is more erratic.

Precise interpretation of the curves is complicated by the apparent overlapping of residual proliferation and initial lysis in the early phase and by the increased significance of a residue of non-lysing cells toward the end. Furthermore, a small amount of secondary growth, suggested in

TABLE II  
*Rate of Bacterial Lysis in Experiment Represented by Fig. 1*

Reading No.	Time		Relative bacterial densities* at time $T_n$				Half time of lysis			
	$T_n$	$\frac{T_n - 1}{T_n - 1}$	200 $\gamma$ lysine	150 $\gamma$ lysine	100 $\gamma$ lysine	50 $\gamma$ lysine	200 $\gamma$ lysine	150 $\gamma$ lysine	100 $\gamma$ lysine	50 $\gamma$ lysine
	hrs.	hrs.					hrs.	hrs.	hrs.	hrs.
1	20		996	769	547	288				
2	23	3	772	604	460	260	8.2	8.6	12.0	20.5
3	29	6	418	351	292	196	6.8	7.5	9.1	14.7
4	40	11	151	163	184	143	7.5	9.9	16.5	24.1
5	48	8	71	86	125	127	7.3	8.7	14.3	47.3
Average.....							7.5	8.7	13	27

\* Obtained by adjusting the net optical densities for deviations from Beer's law. The true relation between net optical densities and relative bacterial densities was obtained by measurements made on serial dilutions; *cf.* Longworth (4).

the 200  $\gamma$  curve of Fig. 1 and very evident when observations are extended for several days, has consistently been encountered. The fact that the amount of secondary growth tends to be larger in the tubes of initially higher lysine content suggests that it is the result of lysine newly available from the breakdown of the lysed cells. If this were the case, and if the sole limiting factor in the secondary growth were available lysine, one could also conclude that the amount of free lysine resulting from the lysis of the original cells is very small. The justification of these conclusions was tested by the following experiment.

In order to verify the growth-supporting capacity of the medium after lysis, 5 cc. of the contents of a tube originally containing 150  $\gamma$  of lysine in the high buffer medium, in which lysis subsequent to initial growth had nearly gone to completion, were added to 5 cc. of a tube containing



150  $\gamma$  of lysine in the low buffer medium. Good growth in this combination (A) indicated that lysis does not impart significant inhibitory properties to the medium. Accordingly, the small amount of growth observed in a parallel experiment (Combination B), in which the 5 cc. of low buffer medium did not contain lysine, indicated that after lysis the medium contains very little available lysine. Each of these combinations was prepared twice independently; the new tubes were freshly inoculated and incubated at 29°. The results are shown in Table III.

A characteristic phenomenon in tubes containing lysine-limited growth in high buffer medium is the appearance, on shaking, of surface foaminess suggestive of dissolved protein. However, the solution resulting from lysis produces no coagulation when boiled, and only a faint precipitate is given with trichloroacetic acid. It should also be mentioned that the cells which are subject to lysis differ from their normal counterparts by the

TABLE III  
*Properties of Medium after Lysis*

Combination	0 hr.	17 hrs.	23 hrs.	41 hrs.
A	78	592	573	492
"	72	622	598	500
B	73	100	82	68
"	76	84	77	72

The results are expressed in terms of optical density.

possession of characteristic agglutinative properties, which are especially apparent in washing operations and which recall the mucoid cell types known to bacteriologists.

#### DISCUSSION

The present evidence merely defines a few of the factors determining bacterial lysis in a lysine-limited medium. A conceivable working hypothesis for further experimentation may draw upon two additional lines of evidence from other sources. One is the recent work of Gale (5) which demonstrates that bacterial cells may contain both protein-bound amino acids and free amino acids, and that the relative amounts of internal free amino acids vary, depending on the nature of the medium. Specifically, Gale showed that cells grown in a lysine-rich medium contain approximately 1 per cent (of their dry weight) of free lysine, while growth in a medium of low lysine concentration (12 to 18  $\gamma$  per cc., *i.e.* similar to our submaximal concentrations) resulted in a free lysine content of only 0.1 per cent. In the light of such findings, it appears that the intracellular reserve of free lysine may be a factor in the differences in bacterial stability

encountered in our work. Some indirect evidence has been obtained which supports this view. As shown above, cells grown in lysine-limited high buffer medium will undergo lysis regardless of the buffer concentration of the medium to which they are transferred. However, when such cells were transferred to 10 cc. of a solution which contained, besides the inorganic salts of the low buffer medium, only 12 mg. each of L-glutamic acid and L-lysine, lysis was completely prevented. This observation may be interpreted in terms of the finding of Gale that, under some conditions, glutamic acid aids the entry of lysine into lysine-deficient cells. Yet, if we consider lysis under our conditions as hypothetical evidence of a deficiency of internal free lysine, our observations differ from those of Gale in important details. Gale readily demonstrated imbibition of lysine even in the absence of glutamic acid. We have been unable to do so. Gale found that cells rich in free lysine will not lose it to a lysine-free medium unless the latter contains glucose and thus supplies glycolytic energy. We have found, to the contrary, that cells grown in the low buffer medium, which do not undergo lysis either in the high or the low buffer medium, will do so in a salt solution free of organic nutrients, and that this lysis is prevented by the addition of glucose only. Similarly, the lysis of cells grown in the high buffer medium was retarded by the addition of glucose only to a salt solution free of organic nutrients. However, comparison of Gale's observations and ours with the same bacterial strain remains to be made.

While Gale's discovery of bacterial free amino acids provides a hypothetical explanation for the different properties of cells grown in the presence and absence of excess lysine, it seems possible to view the singularity of the position of lysine, among other amino acids, as regards bacterial stability, in the light of the pioneer work of Schoenheimer on the rôle of exchange reactions in amino acid metabolism. According to the work of Schoenheimer and his school (6), most amino acids in the proteins of living tissue exchange hydrogen atoms with water molecules and nitrogen atoms with ammonium ions circulating in the tissue, but lysine occupies an exceptional position in that it does not participate in the exchange of either hydrogen or nitrogen. Exchange reactions fail to occur because lysine, unlike other amino acids, cannot be resynthesized from its deamination product, although, in common with other amino acids, it undergoes continuous metabolic deamination. By coupling this finding with the other important discovery of Schoenheimer, the process of continuous metabolic exchange of protein-bound amino acids, one may conclude that, in a biological system of limited amino acid content, lysine will be lost irreversibly through the combined processes of exchange and deamination, while other amino acids will be conserved, comparatively speaking, because of the reversibility of

their deamination. Application of this concept, together with the findings of Gale, to bacteria would imply that the stored lysine of cells grown in the presence of an abundant supply acts as a metabolic reserve and that the absence of such internal reserve in cells grown without excess lysine results in an irreplaceable metabolic loss of lysine with consequent protoplasmic breakdown, *i.e.* lysis. Acceleration of amino acid metabolism by phosphate would also be implicit in such an interpretation.

#### SUMMARY

In cultures of *Streptococcus faecalis* (ATCC No. 9790) in a synthetic medium containing suboptimal concentrations of lysine, initial growth, which is proportional to the available lysine, is followed by rapid lysis of cells if the medium contains 0.30 M phosphate buffer of pH 6.5. The phosphate effect cannot be duplicated by sodium chloride of equal ionic strength or by citrate of equal pH and buffering capacity. Lysis is an expression of properties acquired by cells grown in the specified medium, and not an effect of the medium upon bacterial cells. An excess, beyond the amount required for maximal growth, of 5  $\gamma$  of lysine per cc. is sufficient to prevent lysis. Lysis tends to follow the pattern of a monomolecular reaction, and yields soluble protein-like degradation products and little, if any, lysine. The discussion considers the possible interpretation of these observations in terms of the discovery of Gale of variable quantities of free amino acids in bacteria and of the findings of Schoenheimer on the metabolic exchange of protein-bound amino acids and the irreversibility of the deamination of lysine.

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# HYPERCHOLESTEREMIA IN THE RAT AFTER INTRAVENOUS ADMINISTRATION OF CHOLESTEROL

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(Received for publication, September 4, 1948)

Study of mammalian metabolism of cholesterol has been hampered because of the fact that this substance may be absorbed or synthesized, as well as excreted or destroyed, within the animal body. These processes appear to be so well integrated that, with the exception of the rabbit (10) and the chicken (7), it has been found difficult to change the concentration of cholesterol significantly in the blood of normal animals by oral administration of this substance alone.

One of the simplest means of overcoming the mechanisms of normal balance should be the intravenous administration of cholesterol. However, little can be found in the literature describing a consistent elevation of blood cholesterol to a high level in *omnivorous mammals* by this means. Although Remesow (11) and Remesow and Tavaststyernya (12) reported the production of temporary hypercholesteremia in dogs after intravenous injection of a special cholesterol suspension, subsequent workers (9, 15) were unable to prepare suspensions according to their directions. Other authors (2, 4-6, 14) have found no appreciable rise, or an inconsistent rise, in the blood cholesterol of omnivorous mammals after injection of their own preparations of colloidal cholesterol. Only McKibbin *et al.* (8) have reported single determinations of hypercholesteremia in two dogs following chronic intravenous alimentation with an emulsion containing a fraction of 1 per cent of cholesterol.

Recently the rabbit and chicken have been made hypercholesteremic by intravenous injection of cholesterol. Bevans, Abell, and Kendall (1) succeeded in raising the serum cholesterol of rabbits 300 mg. per cent above the initial concentration by the injection of cholesterol suspension stabilized with sodium stearate. Horlick, Feldman, and Katz (7) were able to increase the blood cholesterol of chickens by the intravenous injection of plasma obtained from other chickens previously made hypercholesteremic by dietary forcing of cholesterol. The hypercholesteremia in these injected chickens consisted preponderantly of esterified cholesterol.

Thus it can be seen that little information is available concerning the

fate of free cholesterol intravenously administered to an omnivorous mammal. Accordingly, it seemed of value to determine the plasma levels of free and esterified cholesterol after intravenous injection of free cholesterol into the laboratory rat.

In order to make such a study, it was necessary to prepare a suspension of cholesterol which could be administered intravenously to the rat, without gross toxic effects, in sufficient concentration and quantity to alter consistently the level of blood cholesterol.

### *Methods*

The cholesterol suspension was prepared as follows: 3 gm. of cholesterol were dissolved in 50 cc. of ether; 1 gm. of lauric acid was dissolved in 100 cc. of 0.05 N NaOH. The ether and watery solutions were mixed by shaking and passed several times through a hand homogenizer of the cylinder and piston type. The white emulsion produced was passed slowly through the apparatus of Cole *et al.* (3), in which ether was removed by steam at atmospheric pressure. The pH was adjusted to 7.4 by addition of a few cc. of a concentrated solution of  $\text{Na}_2\text{HPO}_4$ , with rapid stirring. The resultant suspension was centrifuged for 15 minutes at approximately 4000 R.P.M., or until any sediment was deposited. Frequently no appreciable sediment was observed. The supernatant fluid from this centrifugation was collected and employed for the intravenous injection; the cholesterol content of this type of suspension varied from 2 to 2.8 gm. per 100 cc. Such suspensions occasionally became toxic after storage for 2 weeks or longer, although stored in closed, full containers and under refrigeration.

This cholesterol suspension was injected into eight groups of rats (Wistar and Long-Evans strains), both male and female. Their average weight was 163 gm. (range, 102 to 240 gm.). Before the injection of cholesterol (20 mg. of cholesterol per 100 gm. of weight usually contained in 0.8 to 1.2 cc. of the suspension), control samples of 2 cc. of blood were secured from the majority of the rats. Withdrawal of this amount of blood was found in preliminary studies not to effect any subsequent changes in the blood cholesterol of the rat.

The changes in the free and esterified cholesterol content of rat plasma following such injection were studied by analyses of individual blood samples of each group of rats. A single group of rats was used for the determination of the cholesterol (free and esterified) content of plasma at any given time after injection.

The heparinized plasma of the blood samples was analyzed for total cholesterol and for free cholesterol according to the method of Saifer and Kammerer (13). Ester cholesterol was calculated by difference.

### *Results*

The injection of 20 mg. of free cholesterol per 100 gm. of body weight into the first group of seventeen rats invariably produced an immediate and marked rise in the cholesterol content of their plasma. As can be seen from Table I, this increase consisted largely of free cholesterol, which rose from the average preinjection value of 12 mg. per 100 cc. to 295 mg. per 100 cc. (an increase of over 2300 per cent) 15 minutes after injection of free cholesterol. As might be expected, no rise occurred in the cholesterol ester content at this time. Actually, the average cholesterol ester content was 26 mg. per 100 cc. 15 minutes after injection, a value lower than the preinjection level. Whether this decrease was real or due to a small percentage error in ascertaining large values of total and free cholesterol could not be determined.

This great excess of free cholesterol in plasma decreased sharply, however, for 3 hours after injection of cholesterol into the second group of twenty-two rats (see Table I) the average free cholesterol content was only 64 mg. per 100 cc., an increase of slightly under 400 per cent above the preinjection value (13 mg. per 100 cc.). On the other hand, the cholesterol ester content of this group of rats increased from a preinjection value of 37 mg. per 100 cc. to 58 mg. per 100 cc. (an increase of 57 per cent).

Compared to the blood content of free and esterified cholesterol in rats bled after 3 hours, little or no significant change occurred in the concentration of these substances in groups of rats bled 6 and 12 hours, respectively, after injection (see Table I).

The average free cholesterol content of a group of rats bled 18 hours after injection was 46 mg. per 100 cc. (approximately 283 per cent above the control value of 12 mg. per 100 cc.). The average cholesterol ester content of this same group remained elevated (52 mg. per 100 cc.). The average free cholesterol content of the group of rats bled 24 hours after injection was 27 mg. per 100 cc. (approximately 170 per cent above the preinjection value of 10 mg. per 100 cc.), but the esterified cholesterol content was only 11 per cent above the control value of 44 mg. per 100 cc.). Likewise, the esterified cholesterol content of plasma of the group of rats bled 36 hours after injection approximated the average preinjection level (see Table I). The free cholesterol content of this same group, however, was still 82 per cent higher than the control value (11 mg. per 100 cc.). The cholesterol ester content of plasma of the group of rats bled 48 hours after injection, however, was again rather high (40 mg. per 100 cc.), although whether this represented a physiologically significant elevation could not be determined. The free cholesterol content of this last group of rats, however, was still significantly higher (40 per cent) than the preinjection value.





## DISCUSSION

The preceding experimental observations made it clear that intravenous injection of a suitable suspension of cholesterol was not only feasible but a relatively simple procedure. Furthermore, because the method described allows the intravenous injection of larger or smaller quantities of free cholesterol than actually were given in the present study, a means is available to alter at will the total cholesterol content of the plasma of the rat.

The rapid rise and subsequent gradual fall of free cholesterol, together with the rise in esterified cholesterol in the plasma of rats following the intravenous injection of free cholesterol, indicated to us that this animal was able not only to free its blood of excess free cholesterol but also to metabolize a portion of the latter to the esterified form. The process by which both of these phenomena were effected was not revealed in the present studies. However, it is possible that with the present method of producing an immediate and controlled hypercholesteremia in the omnivorous mammal such processes may be studied.

## SUMMARY

1. The preparation of a cholesterol suspension which may be injected intravenously into rats without gross toxicity is described.

2. An immediate marked rise in plasma-free cholesterol and a gradual fall lasting over 48 hours followed each injection. Cholesterol esters rose moderately while the free cholesterol concentration was falling. Within 36 hours following the injection, however, the cholesterol ester content of plasma had returned approximately to the preinjection level.

The authors wish to express their thanks to Rheta Goldberg for technical assistance.

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## CYTOCHEMICAL STUDIES OF MAMMALIAN TISSUES\*

### II. THE DISTRIBUTION OF DIPHOSPHOPYRIDINE NUCLEOTIDE-CYTOCHROME *c* REDUCTASE IN RAT LIVER FRACTIONS

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(Received for publication, October 4, 1948)

The use of differential centrifugation in the isolation of intracellular components constitutes an important method of approach to the problem relating cell chemistry and cell structure. A number of difficulties have arisen, however, in the interpretation of data obtained by this procedure, one of the most troublesome problems having been the cytological identification of the cellular component or components present in the isolated fractions. In a previous report (1), an improved method was described for the isolation of sedimentable components of rat liver cells. It was demonstrated that by the use of a hypertonic solution of sucrose (0.88 M) as the medium morphologically intact mitochondria could be obtained in good yield and in reasonably homogeneous preparations. A procedure for the isolation of submicroscopic particulate material with the use of this medium was also included. Some of the biochemical properties of mitochondria and submicroscopic particles were described.

These and additional biochemical data obtained in other laboratories have shown that mitochondria play an important rôle in cellular metabolism. It is now well established, for example, that the elements are intimately concerned with cell respiration. Thus the major proportion of the cytochrome oxidase (2, 3) and succinoxidase (1-3) activity of whole liver was recovered in the mitochondrial fraction. Adenosinetriphosphatase was found by Schneider (2) and riboflavin by Price, Miller, and Miller (4) to be concentrated in the fraction. Kennedy and Lehninger (5), in a preliminary note, have stated that all of the demonstrable fatty acid oxidase activity of rat liver can be recovered in mitochondria but have not presented data permitting a direct comparison of the specific activity of mitochondria with that of whole liver. Schneider (6) independently was able to recover approximately 80 per cent of the octanoxidase activity of liver homogenates in the mitochondrial fraction and showed that the specific activity of the fraction was 3 times greater than that of the whole tissue.

\* Aided in part by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

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Much less information is available concerning the biochemical properties of the submicroscopic particulate material of liver cells. Previous studies (1, 7) have demonstrated that pentose nucleic acid is concentrated in this fraction, and recently Omachi, Barnum, and Glick (8) have discovered that an esterase hydrolyzing methyl butyrate is present in submicroscopic particles in high concentration.

The present report deals with the intracellular distribution of the enzyme system catalyzing the transfer of electrons between dihydrodiphosphopyridine nucleotide (DPNH<sub>2</sub>) and cytochrome *c*, thus linking the coenzyme I-dependent dehydrogenases with the cytochrome oxidase system. This enzyme has been called DPN-cytochrome *c* reductase (9), in order to distinguish it from the cytochrome reductase studied by Haas, Horecker, and Hogness (10), and is known to be associated with insoluble tissue particles (11-13). It has not, however, been localized in a specific structural entity of the cell. The experiments described below demonstrate that DPN-cytochrome *c* reductase is associated with both mitochondria and submicroscopic particles and is concentrated to a pronounced extent in the latter cell fraction.

## EXPERIMENTAL

### *Methods*

*Preparation and Fractionation of Liver Homogenates*—Livers were obtained from young, adult, albino rats. The animals were provided with food until the time of each experiment. They were sacrificed by a blow on the head and were then decapitated and allowed to bleed freely. The livers were quickly removed, chilled, and forced through a masher fitted with a 1 mm. mesh wire screen. 5 gm. of liver pulp were then homogenized in an all-glass apparatus (14) in 50 ml. of 0.88 M sucrose solution. The initial steps of the fractionation procedure, including the preparation of the cytoplasmic extract (Fraction E) by means of the removal of free nuclei, residual unbroken liver cells, and red blood cells (Fraction N) and the isolation and resedimentation of mitochondria (Fraction Mw), were carried out as described previously (1). A revision in the earlier procedure, by which submicroscopic particles of a diameter greater than 100 m $\mu$  had been sedimented (1), was necessary in the present experiments, however, because it was found that a considerable proportion of the DPN-cytochrome reductase activity of the homogenate was associated with particles having a diameter less than 100 m $\mu$ . In previous investigations (1), it was shown that a small proportion of the submicroscopic particulate material was sedimented during the initial isolation of mitochondria but remained largely in the supernatant (Fraction Wm (1)) after resedimentation of the mitochondria. Accordingly, Frac-

tion Wm and the supernatant (Fraction S<sub>1</sub> (1)), obtained after the initial sedimentation of mitochondria, were combined. This mixture was then diluted with an equal volume of water in order to reduce the density and viscosity of the sucrose solution and was centrifuged for 2 hours at 41,000 × gravity. By this procedure it was possible to sediment particles of a diameter as small as 50 mμ. The firmly packed, transparent, red pellet was suspended in 0.88 M sucrose (Fraction P). The final supernatant (Fraction S<sub>2</sub>) was water-clear.

The centrifuges and centrifuge tubes employed in the fractionation were the same as those used previously (1). The centrifugal force was calculated for the bottom of the tubes.

*Determination of DPN-Cytochrome c Reductase*—The determination of the DPN-cytochrome c reductase activity of the various liver fractions was carried out spectrophotometrically at 22–24°, essentially according to the method of Potter and Albaum (13). The rate of reduction of cytochrome c on addition of enzyme and DPNH<sub>2</sub> was estimated by absorption measurements at 550 mμ.

The reaction mixtures were made up by adding the following reagents, in order: 0.20 ml. of 0.50 M potassium phosphate buffer (pH 7.6), 0.10 ml. of 0.82 M nicotinamide, water to give a final volume of 3.00 ml., 0.30 ml. of 10<sup>-3</sup> M NaCN, 0.050 to 0.200 ml. of a suitable dilution of each liver fraction, 0.40 ml. of 1.9 × 10<sup>-4</sup> M cytochrome c, and 0.20 ml. of DPNH<sub>2</sub> solution (containing 0.75 mg. of DPNH<sub>2</sub> per ml.). The blank contained all reagents except enzyme. An additional control containing enzyme but no DPNH<sub>2</sub> was not necessary, since at the dilutions employed for the determination of DPN-cytochrome c reductase none of the liver fractions was capable of reducing cytochrome c in the absence of DPNH<sub>2</sub>. On addition of DPNH<sub>2</sub>, the reaction mixtures were quickly transferred to cuvettes and the increase in optical density at 550 mμ followed at 1 minute intervals in the Beckman quartz spectrophotometer. Each liver fraction was assayed at two or more levels of enzyme activity. The specific activity was expressed as micromoles of cytochrome c reduced per minute per mg. of total nitrogen of the fractions.

The optimum concentration of each component of the reaction mixture was determined in preliminary experiments. Nicotinamide was included to prevent destruction of DPNH<sub>2</sub> by the DPN nucleotidase of liver (15), NaCN to inhibit reoxidation of cytochrome c by cytochrome oxidase.

Fig. 1 shows the rate of reduction of cytochrome c by the liver extract (Fraction E) on addition of DPNH<sub>2</sub>. It can be seen that the reaction proceeded linearly with time over a reasonable period and was directly proportional to the enzyme concentration.

DPNH<sub>2</sub> was prepared by the method of Warburg and Christian (16)

either from a sample of "cozymase" obtained from the Schwarz Laboratories and containing approximately 65 per cent DPN or from a sample of DPN purified by counter-current distribution (17) and containing at least 98 per cent DPN. The DPN-cytochrome reductase activity of the liver fractions when the pure compound was used as a substrate was the same as the activity obtained with the crude cozymase preparation. Similar results were also obtained with a sample of  $\text{DPNH}_2$  isolated according to the procedure of Ohlmeyer (18) and containing approximately 70 per cent

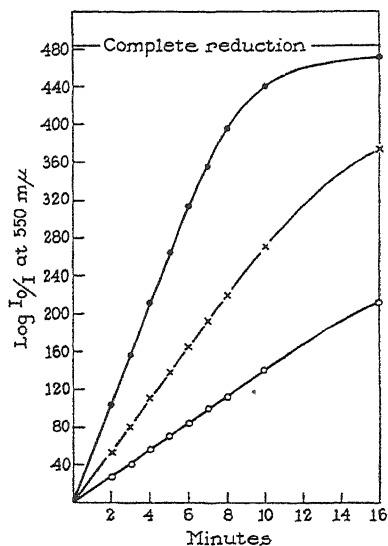


FIG. 1. Reduction of cytochrome *c* by varying amounts of liver extract (Fraction E, diluted 1:200 with 0.88 M sucrose) on addition of dihydridophosphopyridine nucleotide. ●, 0.200 ml. of diluted extract; ×, 0.100 ml. of diluted extract; ○, 0.050 ml. of diluted extract. For the method of preparation of the extract and the components of the reaction mixture, see the text.

$\text{DPNH}_2$ . The  $\text{DPNH}_2$  was kept in 1 per cent  $\text{NaHCO}_3$ -1 per cent  $\text{Na}_2\text{CO}_3$  buffer (16), and the solution was brought to pH 7.6 with 0.5 M  $\text{KH}_2\text{PO}_4$  immediately before use.

Cytochrome *c* was prepared from beef hearts according to the method of Keilin and Hartree (19) and was then reprecipitated from  $(\text{NH}_4)_2\text{SO}_4$  solution with trichloroacetic acid and dialyzed against distilled water. In order to maintain the substance in the oxidized state, the solution was made 0.01 M with respect to HCl. On reduction with hydrosulfite, the increase in optical density at 550  $\text{m}\mu$  of the cytochrome *c* solution corresponded to molecular extinction coefficients of  $0.90 \times 10^7$  sq. cm. per

mole for oxidized cytochrome *c* and  $2.81 \times 10^7$  sq. cm. per mole for reduced cytochrome *c* ((20) p. 190).

*Determination of Total Nitrogen, Pentose Nucleic Acid Phosphorus, and Succinoxidase*—Determination of total nitrogen was carried out colorimetrically after acid digestion of the samples ((20) p. 103). Pentose nucleic acid phosphorus (PNA) was estimated according to the method of Schneider (21), and succinoxidase activity was determined manometrically according to the method of Schneider and Potter (22).

### Results

*Intracellular Distribution of DPN-Cytochrome c Reductase*—Since the presence of clumps of unbroken liver cells and of free nuclei in Fraction N (1) made it difficult to obtain reproducible aliquots at the great dilu-

TABLE I  
*Succinoxidase and DPN-Cytochrome c Reductase Activity of Liver Homogenate and Liver Extract*

Preparation	Experiment No.	Succinoxidase		DPN-cytochrome <i>c</i> reductase	
		Recovery	$Q_{O_2}$ *	Recovery	Specific activity†
		<i>per cent</i>		<i>per cent</i>	
Homogenate	1	100	800	100	2.9
	2	100	800	100	2.7
Liver extract (Fraction E)	1	81	880	88	3.4
	2	76	860	77	3.2

\* Microliters of  $O_2$  per hour per mg. of nitrogen.

† Micromoles of cytochrome *c* reduced per minute per mg. of nitrogen.

tion necessary for the determination of DPN-cytochrome *c* reductase, it was not possible to determine accurately the activity of this fraction. An indirect estimate could be made, however, by determining the amount of enzyme lost from the homogenate on removal of nuclei and residual intact liver cells. On a number of occasions it was found that the recovery of DPN-cytochrome reductase in the liver extract (Fraction E) was similar to the recovery of succinoxidase activity. Table I shows two sample experiments in which simultaneous determinations of the two enzyme systems were made. Since it had been previously shown that the succinoxidase activity of Fraction N was a function of the unbroken liver cells present, this enzyme system apparently not being present in appreciable amounts in nuclei (1), it was concluded from the data in Table I that a similar situation existed with respect to DPN-cytochrome *c* reductase. Thus the distinct rise in the specific activities of the two enzyme systems, after removal of free nuclei and unbroken cells, indicated that

DPN-cytochrome *c* reductase was confined mainly, if not entirely, to the cytoplasm. It was therefore possible in a subsequent investigation of the intracellular distribution of DPN-cytochrome *c* reductase to omit analyses of the homogenate and Fraction N and to work with those fractions containing the cytoplasmic elements of the liver cell (Fractions E, Mw, P, and S<sub>2</sub>).

Table II summarizes the results of four experiments dealing with the distribution of DPN-cytochrome *c* reductase, nitrogen, and pentose nucleic acid phosphorus in the cytoplasm of the liver cell. The over-all recovery of enzyme was considered satisfactory in view of the fact that an average of 95 per cent of the DPN-cytochrome *c* reductase activity of the

TABLE II

*Distribution of Nitrogen, Pentose Nucleic Acid Phosphorus, and DPN-Cytochrome c Reductase in Rat Liver Fractions*

Preparation	Nitrogen		PNA			DPN-cytochrome <i>c</i> reductase	
	Total	Recovery	Total	Recovery	PNA per mg. N	Recovery	Specific activity*
	mg.	per cent	γ	per cent	γ	per cent	
Extract (Fraction E)	85	100	1530	100	18 (17-20)	100	3.5 (3.2-3.8)
Mitochondria (Fraction Mw)	28	33 (29-36)	415	27 (23-33)	15 (13-17)	32 (30-35)	3.5 (3.2-3.9)
Submicroscopic particles (Fraction P)	19	22 (21-25)	870	57 (52-62)	46 (41-49)	58 (55-61)	9.2 (8.1-10.0)
Final supernatant (Fraction S <sub>2</sub> )	38	45 (42-51)	213	14 (12-18)	5.6 (3.9-7.6)	5 (5-5)	0.4 (0.4-0.5)

The figures represent average values; the range of values is given in parentheses.

\* Micromoles of cytochrome *c* reduced per minute per mg. of nitrogen.

liver extract (Fraction E) was accounted for. An average of 90 per cent was associated with sedimentable elements of an approximate diameter of 50 mμ or greater. The mitochondria (Fraction Mw) contained 32 per cent and the submicroscopic particles (Fraction P) 58 per cent, the enzyme system being concentrated to a considerable extent, in terms of total nitrogen, in the latter fraction. Only 5 per cent remained in the final supernatant (Fraction S<sub>2</sub>), an amount that may well have been due to incomplete sedimentation of submicroscopic particles. An idea of the extremely high activity of the enzyme could be obtained from a calculation of the amount of oxygen required to reoxidize cytochrome *c* at the same rate that it was reduced by DPN-cytochrome *c* reductase (1 mole of O<sub>2</sub> being equivalent to 4 moles of reduced cytochrome *c*). Thus a Q<sub>0</sub>,



(microliters of  $O_2$  per hour) of 3100 would be required to compete successfully with the DPN-cytochrome *c* reductase represented by 1 mg. of submicroscopic particulate nitrogen.

*Intracellular Distribution of Pentose Nucleic Acid Phosphorus*—In general, the distribution of PNA in the liver fractions, as shown in Table II, was in agreement with results reported previously (1). Of interest is the fact that both the distribution and concentration of PNA in the fractions were some-

TABLE III

*Effect of Repeated Sedimentation of Mitochondria and Submicroscopic Particles on Total Nitrogen, Pentose Nucleic Acid Phosphorus, Succinoxidase, and DPN-Cytochrome c Reductase*

Fraction	Nitrogen	PNA		Succinoxidase		DPN-cytochrome <i>c</i> reductase	
		Total	PNA per mg. N	Recovery	$QO_2^*$	Recovery	Specific activity†
	mg.	$\gamma$	$\gamma$	per cent		per cent	
M (mitochondria, sedimented once)	35.2	630	17.9	100	1800	100	4.2
Mw <sub>1</sub> (mitochondria, sedimented twice)	26.0	445	17.1	94	2200	70	3.9
Mw <sub>2</sub> (mitochondria, sedimented 3 times)	23.7	326	13.8	87	2400	63	3.9
Mw <sub>3</sub> (mitochondria, sedimented 4 times)	24.7	324	13.1	87	2400	59	3.5
P (submicroscopic particles, sedimented once)	19.6	795	40.6			100	8.8
Pw <sub>1</sub> (submicroscopic particles, sedimented twice)	16.3	708	43.4			93	9.8
Pw <sub>2</sub> (submicroscopic particles, sedimented 3 times)	16.3	721	44.2			84	8.8

\* Microliters of  $O_2$  per hour per mg. of nitrogen.

† Micromoles of cytochrome *c* reduced per minute per mg. of nitrogen.

what similar to corresponding data obtained for DPN-cytochrome *c* reductase.

*Effect of Repeated Sedimentation on DPN-Cytochrome c Reductase Activity of Mitochondria and Submicroscopic Particles*—Although the data in Table II showed that DPN-cytochrome *c* reductase was associated with mitochondria after two sedimentations and with submicroscopic particulate material after a single sedimentation, it was desirable to determine in further experiments whether the enzyme activity could be released from these components of the cytoplasm by repeated sedimenta-

tion and resuspension. Accordingly, mitochondria were isolated and were then subjected to three cycles of sedimentation and resuspension in 0.88 M sucrose. An aliquot was retained for analysis after each resuspension, and the four preparations were analyzed for total nitrogen, PNA, succinoxidase, and DPN-cytochrome *c* reductase. The results of this experiment are shown in Table III. It is apparent that the recovery of nitrogen and PNA reached a constant level after the third sedimentation. The recovery of succinoxidase declined only slightly during the procedure, and there was an appreciable rise in the succinoxidase  $Q_{O_2}$ . These findings were previously (1) shown to indicate practically complete removal from mitochondria of submicroscopic particles and soluble material not associated with sedimentable elements. The ratio, micrograms of PNA to mg. of nitrogen, and the succinoxidase  $Q_{O_2}$  obtained for repeatedly sedimented mitochondria (Fractions  $Mw_2$  and  $Mw_3$ ) were in agreement with values reported earlier (1).

The data in Table III show that the recovery of DPN-cytochrome *c* reductase closely followed that of nitrogen and PNA and reached essentially a constant level after two sedimentations. The slight decline in specific activity on repeated sedimentation of mitochondria was probably due, at least in part, to removal of submicroscopic particles.

Table III also shows the results of a similar experiment in which submicroscopic particles were isolated according to the fractionation procedure described above and then washed twice with 0.44 M sucrose. In general, the data show that two sedimentations sufficed to remove soluble material from the submicroscopic particles, since there was an initial loss in nitrogen and PNA, but the recovery values did not change after a third sedimentation. A decline in the recovery and specific activity of DPN-cytochrome *c* reductase occurred, however, after the third sedimentation and could not be accounted for in an assay of the supernatant. This decline may have been due to denaturation of the enzyme, since the temperature of the centrifuge rotor rose above room temperature during the final centrifugation. The rise in the specific DPN-cytochrome *c* reductase activity after the second sedimentation was probably a reflection of the removal from the submicroscopic particles of the soluble material of the liver cell.

#### DISCUSSION

*Remarks on Method of Determination of DPN-Cytochrome c Reductase—* Several points should be mentioned concerning the method used in the present experiments for determining DPN-cytochrome *c* reductase. Perhaps its most serious disadvantage lay in the fact that it required the use of  $DPNH_2$ , a compound that is not readily obtainable in the pure state, as a

substrate. It was obvious that crude  $\text{DPNH}_2$ , prepared by reduction with hydrosulfite of impure DPN, could be used only when it was demonstrated that the impurities did not affect the enzyme determination. This difficulty was eliminated through the isolation of highly purified DPN by the procedure of counter-current distribution (17). Thus it was shown that the distribution of DPN-cytochrome *c* reductase in the liver fractions and their specific activity were the same with  $\text{DPNH}_2$  prepared from pure DPN as with the impure preparations. It was also possible by other means to obtain good evidence that  $\text{DPNH}_2$  was the only component of the crude preparation that entered into the reaction. By means of simultaneous absorption measurements at both 340 and 550  $m\mu$  and by utilizing the molecular extinction coefficients of  $\text{DPNH}_2$  (18) and reduced cytochrome *c* (19) at these wave-lengths, it was found that the rate of oxidation of  $\text{DPNH}_2$  corresponded stoichiometrically to the rate of reduction of cytochrome *c* by the liver enzyme (1 mole of  $\text{DPNH}_2$  being equivalent to 2 moles of cytochrome *c*).<sup>1</sup> In additional experiments, limiting amounts of  $\text{DPNH}_2$  and an excess of cytochrome *c* were added to the reaction mixture, and the reaction was allowed to proceed until the optical density at 550  $m\mu$  reached a constant level. The amount of cytochrome *c* converted to the reduced state corresponded, according to theory, to the amount of  $\text{DPNH}_2$  added, the latter quantity having been calculated from the optical density at 340  $m\mu$  of the solution of crude  $\text{DPNH}_2$ . From these experiments it was also apparent that the reaction went to completion in the presence of a limiting amount of  $\text{DPNH}_2$ .

Other methods of determining DPN-cytochrome *c* reductase, involving specific enzymatic reduction of DPN, were not considered suitable for the present study because of complications introduced by the necessary addition of further substrates and enzymes to the reaction mixture. Potter (9) has published a method for the assay of the enzyme in tissue homogenates through the malic dehydrogenase system. The method involves the addition of excess malic dehydrogenase, malate, and glutamate and depends upon the finding that transaminase and cytochrome oxidase are apparently present in homogenates in greater quantity than DPN-cytochrome *c* reductase. This system would not be practicable for use in the study of isolated cell components, however, because the distribution among cell fractions of DPN-cytochrome *c* reductase, cytochrome oxidase, and transaminase is not the same. Cytochrome oxidase is associated with mitochondria and not

<sup>1</sup> These experiments were somewhat complicated by the fact that cytochrome *c*, on being reduced, showed a progressive but relatively small decline in absorption at 340  $m\mu$ . Before the rate of oxidation of  $\text{DPNH}_2$  could be determined accurately by absorption measurements at this wave-length, it was first necessary to reduce cytochrome *c* through the succinic dehydrogenase system and standardize the decline in absorption at 340  $m\mu$  in terms of the increase in absorption at 550  $m\mu$ .

present in submicroscopic particles (3), whereas transaminase, on the basis of preliminary experiments,<sup>2</sup> is present mainly in the final supernatant (Fraction S<sub>2</sub>). Further, under the conditions of the DPN-cytochrome *c* reductase determination employed in the present investigation, the enzymatic reduction of cytochrome *c* by the liver extract (Fraction E) proceeded at a more rapid rate than the reoxidation of cytochrome *c* by cytochrome oxidase. Thus in several experiments, the rate of reduction of cytochrome *c* by liver extracts on addition of DPNH<sub>2</sub> and in the absence of cyanide was 63 to 66 per cent of the rate in the presence of 10<sup>-4</sup> M cyanide.

*Intracellular Distribution of DPN-Cytochrome c Reductase*—The results summarized in Tables II and III clearly showed that the DPN-cytochrome *c* reductase of rat liver was associated with sedimentable components of the cell and was not released from these elements on repeated sedimentation and resuspension. As far as could be determined, both mitochondria and submicroscopic particles possessed the enzyme system, the concentration in terms of total nitrogen being much higher in the submicroscopic particles. The latter finding was of interest in view of the fact that only two other biochemical properties can at present be clearly ascribed to this fraction; namely, pentose nucleic acid (1) and an esterase (8). The significance of the high concentration of DPN-cytochrome *c* reductase in the submicroscopic particles will become apparent only when further properties of the fraction have been discovered and when further information is available concerning the intracellular distribution of enzymes capable of reducing DPN.

As is shown in Table II, the concentration of DPN-cytochrome *c* reductase in mitochondria was approximately the same as in the liver extract (Fraction E). Past experience with the technique of cell fractionation led to the opinion that data indicating the presence of an enzyme in a cell fraction should be interpreted with caution unless the enzyme is concentrated in the fraction to a considerably greater extent than in the original starting material. More recently, however, it was possible to isolate morphologically intact mitochondria from rat liver in excellent yield and to show, on the basis of cytological observations and biochemical data, that the preparations contained few, if any, extraneous cellular components (1). In view of the latter findings, it was concluded from the data in Tables II and III that DPN-cytochrome *c* reductase is a function of mitochondria as well as of submicroscopic particles. It does not seem surprising, in fact, that at least a portion of the DPN-cytochrome *c* reductase should be structurally associated with the cytochrome oxidase system and thus present in the mitochondrial fraction. In support of this idea, the finding that cytochrome *c* was reoxidized by liver extracts in the absence

<sup>2</sup> Hogeboom, G. H., unpublished experiments.

of cyanide at a slower rate than it was reduced by DPN-cytochrome *c* reductase may well be due to the fact that only a part of the latter system is in close structural association with cytochrome oxidase.

#### SUMMARY

Homogenates of rat liver were prepared in 0.88 M sucrose solution and fractionated by means of differential centrifugation. A study was made of the distribution among the various liver fractions of the enzyme system catalyzing the transfer of electrons between dihydrodiphosphopyridine nucleotide and oxidized cytochrome *c* (DPN-cytochrome *c* reductase). The distribution of pentose nucleic acid phosphorus (PNA) was also determined. The following results were obtained.

1. The removal of free nuclei from liver homogenates was attended by an increase in the specific DPN-cytochrome *c* reductase activity, a finding which indicated that the enzyme system was confined mainly to the cytoplasm of the liver cell.

2. Of the total DPN-cytochrome *c* reductase activity of liver extracts containing the components of the cytoplasm, 32 per cent was recovered in the mitochondrial fraction and 58 per cent in submicroscopic particulate material of an approximate diameter of 50 to 150 m $\mu$ . An additional 5 per cent remained in the final supernatant obtained after sedimentation of the submicroscopic particles.

3. DPN-cytochrome *c* reductase was concentrated to a pronounced extent, in terms of total nitrogen, in submicroscopic particles. The average specific activity (micromoles of cytochrome *c* reduced per minute per mg. of nitrogen) of this fraction was 9.2, as compared with values of 3.5 for the cytoplasmic extract, 3.5 for mitochondria, and 0.4 for the final supernatant. Release of the enzyme system from submicroscopic particles and mitochondria could not be effected by repeated sedimentation and resuspension of these cellular elements.

4. The distribution of pentose nucleic acid phosphorus among the liver fractions was somewhat similar to that of DPN-cytochrome *c* reductase. Of the total PNA of the liver extracts, 27 per cent was recovered in mitochondria, 57 per cent in submicroscopic particles, and 14 per cent in the final supernatant.

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## DETERMINATION OF ALDOLASE IN ANIMAL TISSUES\*

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(Received for publication, October 7, 1948)

Aldolase catalyzes the reversible cleavage of 1 mole of fructose-1,6-diphosphate (HDP) into 1 mole of glyceraldehyde-3-phosphate and 1 mole of dihydroxyacetone phosphate (1, 2). Although the enzyme has been obtained in crystalline form by Warburg and Christian (3) and by Taylor, Green, and Cori (4) only fragmentary data concerning the content of aldolase in different mammalian tissues have been reported (1, 5, 6). Meyerhof and Lohmann measured the activity of the enzyme by following the formation of the alkali-labile phosphate of the triose phosphates (1). This method was also used by Herbert *et al.* (7) in purification of the enzyme. Although this method, when employed under proper conditions, is perfectly reliable, it is inconvenient, owing to the necessity of performing many blank determinations of inorganic and alkali-labile P. Warburg and Christian employed a spectrophotometric test based on the reduction of diphosphopyridine nucleotide (DPN) which occurs when glyceraldehyde phosphate, formed from HDP by aldolase, is allowed to react with DPN in the presence of crystalline triose phosphate dehydrogenase and arsenate (3). This method does not lend itself to routine use, owing to its technical requirements. Furthermore, as will be shown, it has been found to be subject to large negative errors when used in assaying certain crude tissue extracts, owing to the presence of enzymes capable of destroying DPN. More recently, Dounce and Beyer have published details of a method (6) of assaying aldolase based on colorimetric determination of trioses by the Barker and Summerson procedure (8) for determination of lactic acid. Although the method appears to be quite reliable with crystalline aldolase, its application to crude tissue extracts or homogenates yields much less certain results, as the authors have pointed out.

In the course of a reexamination of the findings of Warburg and Christian concerning serum aldolase levels in tumor-bearing rats (5) a method for quantitative determination of aldolase was devised which is free from some of the difficulties of the several methods outlined above and which allowed a quantitative survey of the enzyme in rat tissues to be made.

*Principle of Method*—HDP is incubated with the buffered test sample

\* This investigation was supported by grants from the American Cancer Society (recommended by the Committee on Growth of the National Research Council) and from Mr. Ben May, Mobile, Alabama.

and hydrazine to fix the triose phosphates formed. At the end of the incubation period the reaction is stopped with trichloroacetic acid and an aliquot of the filtrate is then treated with alkali, followed by acid 2,4-dinitrophenylhydrazine. On making the mixture alkaline again, a characteristic color appears, with maximum absorption at 540 m $\mu$ , owing to the formation of a 2,4-dinitrophenylhydrazine derivative of the triose. The intensity of the color is directly proportional to the enzyme concentration.

*Reagents—*

*Fructose-1,6-diphosphate*, 0.05 M, pH 8.6. Commercial material (Schwarz Laboratories<sup>1</sup>) in the form of the barium salt is purified by the method of Neuberg, Lustig, and Rothenberg (9). Barium is removed from a solution of the purified HDP in 1 M HCl by addition of the required amount of Na<sub>2</sub>SO<sub>4</sub>. If the solution has a yellow color, this may be removed by a single shaking with norit. The solution is then adjusted to pH 8.6 by the addition of sodium hydroxide. The solution is then filtered and diluted to make a concentration of 0.05 M. Since losses of HDP occur in the removal of barium, we have determined the HDP content prior to dilution by use of the fructose method of Roe (10), assuming that 1 mole of HDP is equivalent to 0.525 mole of fructose. This HDP solution is kept refrigerated and is stable for at least 2 weeks.

*Hydrazine*, 0.56 M, pH 8.6. Merck's hydrazine sulfate, brought to pH 8.6 with NaOH and diluted to make a 0.56 M concentration.

*Tris(hydroxymethyl)aminomethane buffer*, 0.1 M, pH 8.6 (11). When the commercial product is used without recrystallization, it has a yellow color which is readily removed by shaking the alkaline solution with norit. It is then adjusted to pH 8.6 with hydrochloric acid and made to volume. This solution is kept in the cold.

*2,4-Dinitrophenylhydrazine*. 1 gm. of 2,4-dinitrophenylhydrazine is dissolved in 1.0 liter of 2 N HCl and the solution filtered.

*Sodium hydroxide*, 0.75 N solution.

*Trichloroacetic acid*, 10 per cent solution.

*Procedure*

The sample to be assayed is added to a test-tube in a bath at 38° containing 1.0 ml. of buffer, 0.25 ml. of HDP solution, 0.25 ml. of hydrazine solution (hydrazine and buffer may be dispensed as a combined solution), and sufficient water to make the total volume 2.5 ml. 30 minutes after

<sup>1</sup> This material is perfectly adequate for use in the assay method without further purification. However, it contains too much inorganic phosphate to be used as a substrate when measurements of alkali-labile P must be made to standardize the method, as described later. Once a standard curve has been prepared, the Schwarz material may be used routinely without purification.



the addition of the enzyme the reaction is stopped by the addition of 2.0 ml. of 10 per cent trichloroacetic acid. For each enzyme sample a colorimeter blank is prepared and incubated in the same way, except that the HDP is not added until *after* the reaction has been stopped with trichloroacetic acid.

The tubes are centrifuged to separate precipitated protein. 1 ml. aliquots of the supernatants are transferred to colorimeter tubes. 1 ml. of 0.75 N sodium hydroxide is added to each tube and the mixture is allowed to stand for 10 minutes at room temperature. 1 ml. of 2,4-dinitrophenylhydrazine solution is then added to each tube and the tubes placed in the 38° water bath for 10 minutes.<sup>2</sup> Sufficient 0.75 N NaOH (7.0 ml.) is added to give a total volume of 10.0 ml. 10 minutes after addition of alkali the per cent transmission is determined in a photoelectric colorimeter with a 540 m $\mu$  filter. With the Evelyn instrument the blank tube (HDP added *after* trichloroacetic acid) is set at 100 per cent transmission and the transmission of the experimental tube obtained. If the tube gives less than 10 per cent transmission, the color development can be repeated on another but smaller aliquot of the trichloroacetic acid filtrate, the volume of the sample being made to 1.0 ml. with 4.5 per cent trichloroacetic acid.

*Standardization of Method*—Since the chromogen measured in the reaction is of unknown structure (see below) and since pure samples of the triose phosphates are not commonly available for use as primary standards, a standard curve relating per cent transmission to the amount of HDP substrate split is prepared in the following manner. Two or three levels of a proper dilution of some source of aldolase, together with their blanks, are carried through the analysis as described above and per cent transmission readings on 0.5 ml. aliquots obtained. On separate aliquots of the trichloroacetic acid supernatants the alkali-labile P liberation is determined by measuring inorganic phosphate in two 1.0 ml. aliquots, one of which has been incubated in 1 N NaOH at room temperature for 20 minutes and neutralized. The amounts of alkali-labile phosphate of the triose phosphates formed by the enzyme are then used as a primary standard against which the optical density of triose chromogen is standardized. We have chosen to express aldolase activity in terms of c.mm. of HDP split at 38° per hour to facilitate comparison with the commonly used *Q* notation, where  $Q_{\text{substrate}} = (\text{c.mm. of substrate acted on})/(\text{mg. of dry weight} \times \text{time in hours})$  at 38°. In order to convert galvanometer readings on 1.0 ml. aliquots of the trichloroacetic acid filtrate into this expression, we have transformed micrograms of alkali-labile P liberated per aliquot meas-

<sup>2</sup> With high levels of enzyme concentration, the 2,4-dinitrophenylhydrazine derivative of triose will sometimes precipitate but it will redissolve in the alkali added in the next step.

ured into c.mm. of HDP liberated per hour for the total reaction mixture (the actual incubation period is 30 minutes). For this calculation 22.4 c.mm. of HDP = 1 micromole of HDP = 2 micromoles of triose phosphate = 2 micromoles of alkali-labile P = 62.0  $\gamma$  of alkali-labile P. The galvanometer readings of the triose chromogen per 1 ml. of aliquot are then plotted against c.mm. of HDP split, calculated from the alkali-labile P liberated as outlined above, yielding a standard curve such as that shown in Fig. 1.

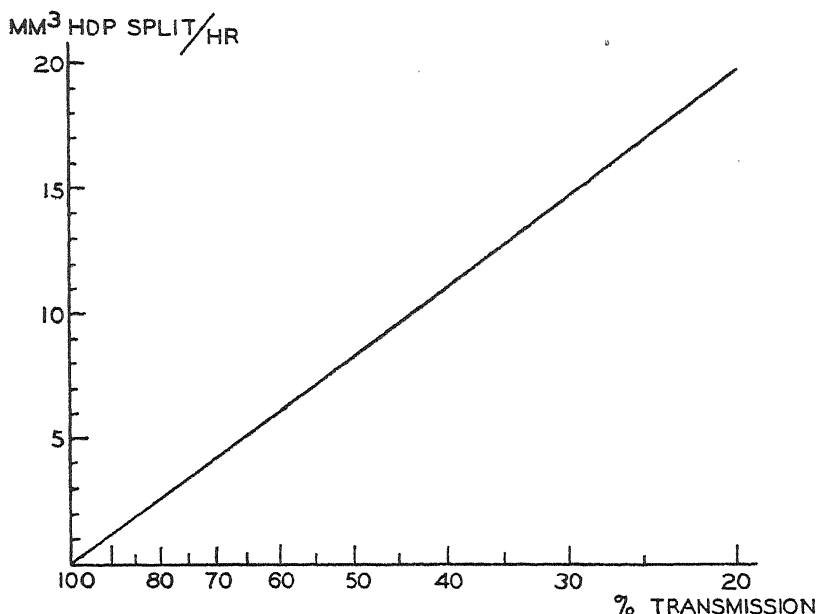


FIG. 1. Standard curve for conversion of per cent transmission readings of triose chromogen into c.mm. of HDP split. This curve was constructed from measurements of triose chromogen and formation of alkali-labile P of the trioses formed during incubation of different levels of enzyme. The per cent transmission readings are actually made on 1.0 ml. aliquots of the trichloroacetic acid filtrate following 30 minutes incubation; the number of c.mm. of HDP split read from the other axis refers to the *total* amount split in the incubation over 1 hour.

Once this standard curve has been prepared, it is unnecessary to re-standardize the method with each set of determinations in these absolute terms, since the relationship between the optical density of the triose chromogen and alkali-labile P liberation has been found to be constant and independent of small variations in different batches of reagents and also independent of the source of enzyme assayed. If measurement of the enzyme in absolute terms is not essential, this standardization is unneces-

sary and an arbitrary scale may be readily defined on the basis of extinction. For purposes of orientation a rat liver homogenate may be used as a temporary semiquantitative standard. Data on the aldolase content of rat liver are given in Table I, and appropriate sample sizes may be calculated readily from the data given.

In order to demonstrate the quantitative and linear relationship between optical density of the triose chromogen and liberation of alkali-labile

TABLE I  
*Aldolase Content of Normal Rat Tissues*

Values expressed as c.mm. of HDP split per gm., wet weight, per hour at 38°.

Tissue	No. of specimens	Range	Mean	Approximate* QHDP
Skeletal muscle . . . . .	12	52,400-105,000	74,800	374
Brain . . . . .	11	11,100- 19,000	15,800	79
Heart . . . . .	11	12,000- 19,000	15,600	68
Liver . . . . .	11	8,500- 14,700	12,100	61
Red marrow . . . . .	3	7,100- 12,100	9,500	48
Adrenal . . . . .	3	5,500- 11,200	8,600	43
Kidney . . . . .	12	6,000- 10,600	7,800	38
Spleen . . . . .	11	3,100- 6,400	4,800	24
Thyroid . . . . .	3	4,400- 5,500	4,800	24
Thymus . . . . .	3	4,200- 5,300	4,700	24
Ventral prostate . . . . .	2	3,700- 5,100	4,400	22
Parotid . . . . .	2	3,300- 4,300	3,800	19
Stomach . . . . .	5	2,400- 5,300	3,700	19
Urinary bladder . . . . .	2	2,000- 5,100	3,200	16
Placenta . . . . .	1		3,000	15
Testis . . . . .	6	2,500- 3,400	2,900	15
Lung . . . . .	9	1,800- 3,700	2,800	14
Uterus . . . . .	1		2,100	11
Erythrocytes . . . . .	3	700- 1,100	900	5
Pancreas . . . . .	8	400- 800	500	3
Fat . . . . .	2	300- 600	400	2
Serum . . . . .	57†	40- 90	60	0.3

\* Assuming that 1 gm. of wet weight = 0.200 gm. of dry weight of the tissue.

† Extensive data on serum are published elsewhere (18).

P, we have plotted in Fig. 2 the per cent transmission of triose chromogen against liberation of alkali-labile P in identical aliquots of the trichloroacetic acid filtrates, using several different levels of enzyme and widely different sources of enzyme. The enzyme sources used were crystalline aldolase (4), an aqueous extract of rabbit muscle acetone powder, a dilute homogenate of rat liver in water, and the blood serum of a sarcoma-bearing rat. The points corresponding to different levels of enzyme over a

wide range, with use of crystalline aldolase or crude materials containing the enzyme, fall on a straight line when per cent transmission of the triose chromogen is plotted on a semilog scale against micrograms of triose P liberated. This line establishes the validity of using the triose chromogen as a measure of triose phosphate formed during the action of aldolase.

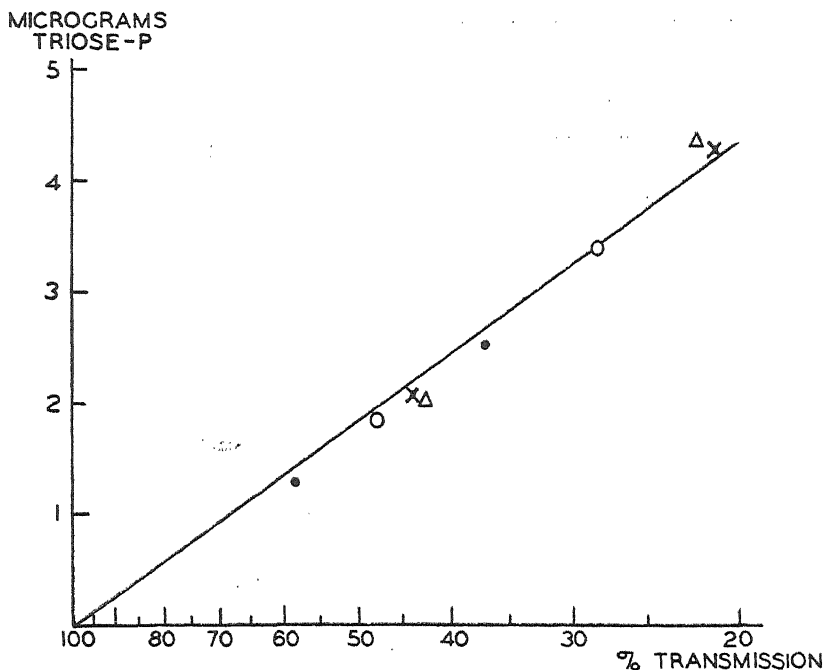


FIG. 2. Proportionality between triose chromogen formed and alkali-labile P formed by various sources and levels of aldolase. Per cent transmission readings of triose chromogen and amount of alkali-labile P formed in 1.0 ml. aliquots of the trichloroacetic acid filtrate. Sources of aldolase, ○ = crystalline aldolase, Δ = aqueous extract of rabbit muscle acetone powder, × = rat liver homogenate, ● = blood serum of tumor-bearing rat.

We have found no significant deviations from this curve in a variety of materials.

*Discussion of Analytical Conditions*—The method of determination of aldolase described was chosen after establishing optimum conditions of pH, substrate concentration, temperature, hydrazine concentration, and of color development. Under these optimum conditions the response given is directly linear with enzyme concentration over a wide range. The course of enzyme action with time is also linear over a wide range of enzyme

concentration well beyond the 30 minute incubation period chosen. The optimum conditions found will be discussed briefly.

The optimum pH was found to be about 8.5 to 9.0. The pH-activity curve in Fig. 3 was constructed from data obtained with a standard amount of enzyme in the incubation system buffered with phosphate in the range up to pH 7.4, with tris(hydroxymethyl)aminomethane from pH 7.4 to 10.5, and with borate at pH 11.0. The optimum so obtained agrees with that found by Herbert *et al.* (7). Dounce and Beyer report a pH optimum of 6.7 when their method was used with crystalline aldolase (6).

Substrate concentration was also varied systematically. The results are represented in Fig. 4. Approximation of the Michaelis-Menten con-

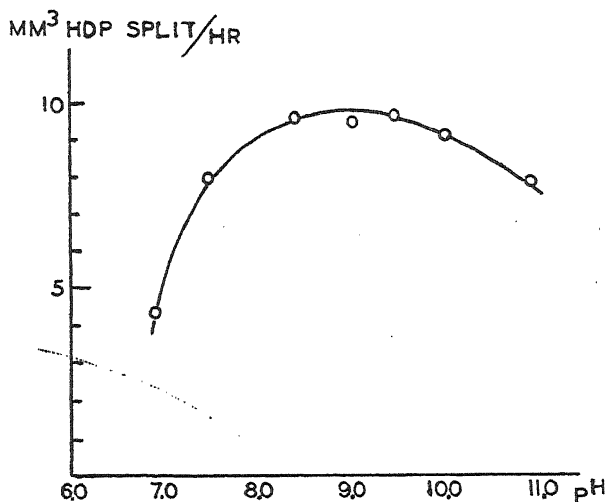


FIG. 3. pH-activity curve of aldolase

stant from these data yields  $K_m = 0.001$  M, in agreement with the figure reported by Herbert *et al.* (7). In the assay system HDP concentration was kept at 0.005 M.

The temperature chosen is 38°. This is not the temperature of maximum activity of highly purified aldolase, which is above 50° (7), but it does give nearly maximum activity with crude homogenates, which contain factors affecting the stability of aldolase at more elevated temperatures.

The time interval chosen for the assay (30 minutes) is within the linear portion of a time-activity curve shown in Fig. 5.

The relationship between HDP split and enzyme concentration under the conditions adopted is linear over a wide range. In Fig. 6 is shown a line obtained with different amounts of an aqueous extract of rabbit mus-

cle acetone powder. An equally linear relationship holds for tissue homogenates.

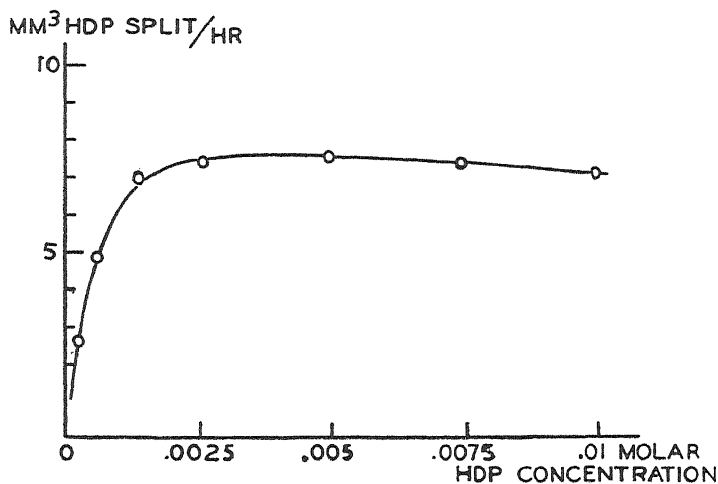


FIG. 4. Effect of substrate concentration in aldolase assay

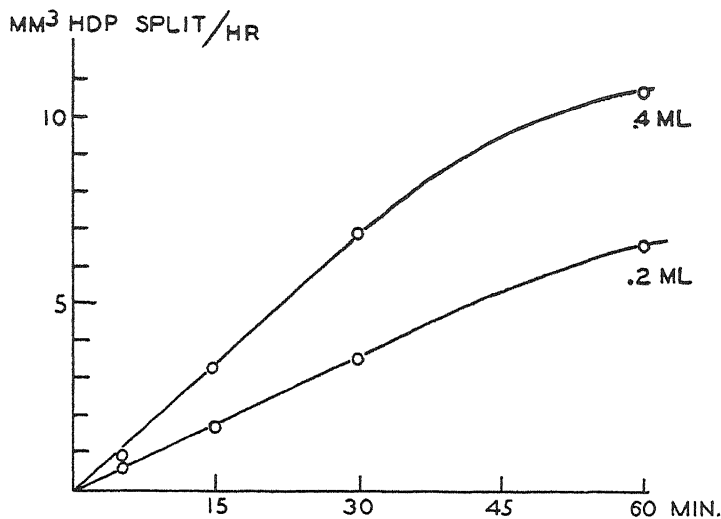


FIG. 5. Time course of aldolase activity in assay

*Nature of Chromogen*—Although the structure of the triose-2,4-dinitrophenylhydrazine derivative responsible for the color reaction is not known a number of facts concerning the chemistry of the reaction have been collected.

It has been found, for instance, that hydrazine is necessary as a fixative for the triose phosphates. However, its rôle is not to maintain linearity of enzyme action with time by removing the products of the reversible aldolase reaction. Actually the amount of HDP substrate split in the assay is less than 1 per cent. However, the hydrazine is required to fix the two triose phosphates as hydrazones in the ratio of 1:1 as they are formed (2), thereby preventing the enzymatic conversion of glyceraldehyde phosphate to dihydroxyacetone phosphate through the action of triose phosphate isomerase (12), which has a wide distribution in animal tissues. The isomerase equilibrium lies overwhelmingly in favor of the formation of dihydroxyacetone phosphate from glyceraldehyde phos-

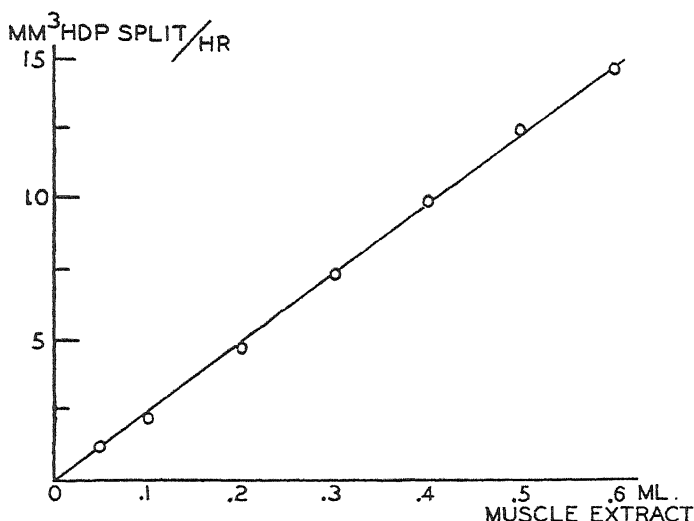


Fig. 6. Linearity of response with enzyme concentration

phate. Obviously if one of the trioses is the chromogen and the other is not chromogenic, then fixation of the triose phosphates in an equimolecular mixture as hydrazones would be necessary to prevent further and variable action of whatever isomerase is present in tissue extracts.

That these considerations hold for this case is indicated by the following experiments. Duplicate samples of crystalline aldolase containing essentially no isomerase were incubated with substrate and buffer in two tubes, one containing hydrazine as usual and the other without hydrazine. At the end of the incubation period the reaction was stopped with trichloroacetic acid and an identical amount of hydrazine was added to the tube which did not contain hydrazine during the enzyme reaction. Colors were then developed from aliquots of the supernatants from both tubes. The per

cent transmission readings of the two samples were identical. Therefore, in the absence of isomerase hydrazine is unnecessary during the incubation. However, if this experiment is repeated with, as the source of aldolase, muscle extract or liver homogenate which contains triose isomerase, instead of the crystalline aldolase, which contains but trace amounts of isomerase, then the tube incubated *without* hydrazine always shows considerably more color than the tube containing hydrazine during the enzyme reaction. Since the presence of triose isomerase in the latter experiments would cause glyceraldehyde phosphate to be converted to dihydroxyacetone phosphate in the absence of a carbonyl fixative, it would appear that dihydroxyacetone phosphate contributes more heavily to color formation than does glyceraldehyde phosphate. To check this assumption duplicate samples of crystalline aldolase were incubated with substrate and buffer (hydrazine was absent from both tubes during the incubation, but added after trichloroacetic acid). To one tube was added an excess of a partially purified preparation of triose phosphate isomerase (12). Colors were then developed from aliquots of the trichloroacetic acid supernatants of each tube. The tube containing added isomerase showed the formation of 1.72 times as much chromogen as the tube without added isomerase. This ratio could not be exceeded by further addition of isomerase. Successively smaller amounts of isomerase produced amounts of chromogen, which declined to the values of the tube without isomerase. It is therefore concluded that dihydroxyacetone phosphate contributes much more heavily to the color formed than does glyceraldehyde phosphate. It is probable from this experiment and other considerations below that glyceraldehyde phosphate also contributes to the color, since, if it did not, an excess of isomerase should have converted essentially all of the glyceraldehyde phosphate to dihydroxyacetone phosphate, which should therefore result in twice the amount of chromogen rather than 1.72 times as much. For these reasons it is clear that hydrazine must be used to fix the two triose phosphates in equimolecular ratio as they are formed to prevent further enzymatic reactions leading to deviations in the yield of dihydroxyacetone phosphate from the HDP split.

The method adopted requires a short period of incubation of the triose phosphate hydrazones in alkali prior to treatment with 2,4-dinitrophenylhydrazine. This treatment has been found to remove the esterified phosphate from the trioses. It is possible that this treatment may also cause other changes in the configuration of the triose hydrazones. If pretreatment with alkali is omitted from the procedure, the colors developed fade within a minute and are of no analytical usefulness. After pretreatment with alkali fading is very slow.

The treatment with 2,4-dinitrophenylhydrazine probably results in the formation of 2,4-dinitrophenylosazones or bishydrazones, since the iso-



lated chromogen (see below) gives a characteristic purple color with alcoholic KOH.

Dihydroxyacetone (recrystallized commercial material) carried through the stage of color development gives a color which is identical in spectrum and stability with the color given by the aldolase reaction product. It gives about 80 per cent of the intensity of color produced by the total trioses formed during enzyme incubation on a molar basis; DL-glyceraldehyde treated in the same way yields a color with a broad maximum at 490  $m\mu$ . At 540  $m\mu$  the intensity on a molar basis is about 25 per cent that of dihydroxyacetone. These findings are in agreement with the idea, expressed above, that both trioses contribute to the color but that dihydroxyacetone is much more active in this respect.

The 2,4-dinitrophenylhydrazine derivative of the triose chromogen has been obtained in crystalline form from large scale enzyme reaction mixtures. As has been mentioned, with higher enzyme activities this derivative precipitates in acid solution. Pooled precipitated material was washed with 0.1 N HCl, dried *in vacuo*, and crystallized from hot 25 per cent nitrobenzene-75 per cent toluene mixtures with norit. Two further recrystallizations yielded fibrous, bright orange needles which were washed with toluene and dried *in vacuo*. The dry compound began to decompose at 265° (block). Microanalysis gave C 43.14 per cent, H 3.44 per cent, N 25.66 per cent. There was no phosphorus present. The compound gave an intense purple with alcoholic KOH, indicative of an osazone, and with aqueous NaOH yielded a color having a broad maximum at 540  $m\mu$ . The substance gives a blue lake with  $Mg^{++}$  and dilute bases, resembling that given by certain azo compounds such as *p*-nitrobenzeneazo- $\alpha$ -naphthol and Magneson (*p*-nitrobenzeneazoresorcinol) (13). This finding appears to be of some significance, since a variety of known 2,4-dinitrophenylhydrazones and osazones we have tested do not give this reaction.

The elementary analysis of the material isolated does not correspond to the empirical formula of the osazones of triose or methylglyoxal or to any compound that we have postulated might be involved. It is possible that the compound is not pure because of considerations already outlined and also of the fact that 2,4-dinitrophenylosazones and hydrazones in general are all readily recrystallized from the solvent used. No other solvents tested were found suitable for recrystallization of the triose chromogen.

Whatever the nature of the chromogen compound, it is clear from the experiments outlined that its measurement is a valid gage of aldolase activity.

*Assay of Normal Rat Tissues*—Meyerhof and Lohmann presented some semiquantitative data showing that aldolase was present in a variety of

animal tissues (1). Warburg and Christian presented data on a few rat tissues obtained with the spectrophotometric assay (5), and Dounce and Beyer gave data on muscle, kidney, spleen, liver, and cell nuclei obtained with their colorimetric method (6).

We have made a survey of the aldolase content of normal rat tissues, using the method presented in this paper.

Organs of decapitated and bled, mature albino rats (heterogeneous colony) were quickly removed and chilled on cracked ice. Samples of approximately 100 mg., wet weight, were weighed on a torsion balance and homogenized in 5.0 ml. of cold water. The homogenate was then diluted to an appropriate volume with cold water. Such dilute homogenates were stable for many hours in the cold. Aliquots of these dilute homogenates were carried through the analysis as described.

The results of these tissue assays are shown in Table I. The aldolase activities are expressed as c.mm. of HDP split per gm., wet weight, per hour at 38°. Dry weights were not determined, but by dividing the values given by 200, they are converted into  $Q_{\text{HDP}} = (\text{c.mm. of HDP split at } 38^\circ) / (\text{mg. of dry weight per hour})$ , assuming that the dry weight = 20 per cent of the wet weight in all of the tissues. This facilitates comparison with corresponding  $Q$  values for other enzymes, such as cytochrome oxidase, succinoxidase (14), etc.

As expected, due to its high glycolytic activity, skeletal muscle showed the highest content, equivalent to  $Q_{\text{HDP}} = 374$ . The single value reported for rat skeletal muscle by Warburg and Christian (5), approximated as  $Q_{\text{HDP}}$ , is 675 at the upper limit of the normal range found by the method used here.

Although the agreement on rat skeletal muscle by this method and the value obtained by Warburg and Christian is fairly good, there is a great difference in values for rat liver and kidney, which Warburg and Christian reported contained very little aldolase and which our method indicates to contain considerable aldolase, about one-sixth that of muscle. In order to resolve this difference the aldolase content of rat liver homogenates was also determined by measuring the liberation of alkali-labile phosphate from HDP in separate aliquots. Values obtained in this way agreed with the values obtained by the method described (see also Fig. 2). It appeared likely that the method of Warburg and Christian is subject to negative errors due to enzymatic destruction of DPN by enzymes present in crude liver and kidney extracts (15, 16). To test this possibility, dihydridiphosphopyridine nucleotide ( $\text{DPNH}_2$ ) of 71 per cent purity (17) was added to the test system of Warburg and Christian (triose phosphate dehydrogenase and HDP were omitted). Rat liver extract was added to the system in a concentration which should have

produced readily measurable reduction of DPN in 3 minutes under their conditions, assuming that our value of the aldolase content of rat liver is correct. Under these conditions the high absorption of  $\text{DPNH}_2$  at  $340 \text{ m}\mu$ , measured in the Beckman spectrophotometer, declined rapidly at a rate about one-half of the expected rate of formation of  $\text{DPNH}_2$  by the amount of aldolase present in the sample. This experiment indicates that there are factors present in crude rat liver homogenates capable of destroying or reoxidizing  $\text{DPNH}_2$  at a rate of the same magnitude as the expected rate of reduction of DPN caused by the combined action of aldolase and triose phosphate dehydrogenase. The method of Warburg and Christian is therefore not suitable for assay of aldolase in crude extracts or homogenates of certain tissues, such as rat liver.

Dounce and Beyer have also reported very low values for liver and kidney compared to muscle (6). However, their method, which was not standardized against a method of known reliability, employs HDP as substrate without a carbonyl fixative. Under these conditions there is no assurance that the triose phosphates do not undergo further enzymatic reactions when homogenates are assayed.

In general the aldolase activity of the different tissues is quite high on a  $Q$  basis, indicating considerable reserve aldolase above that required to account for known rates of carbohydrate metabolism. Of the major organs, pancreas shows a strikingly low content of aldolase.

The findings of Warburg and Christian (5) concerning the elevation of aldolase in the serum of tumor-bearing rats have been reexamined in detail by the method described. The results will be published elsewhere (18).

#### SUMMARY

A method for the determination of aldolase in animal tissues has been described. The method depends on the colorimetric determination of triose formed from fructose-1,6-diphosphate and is free from some of the errors and inconveniences of the techniques previously used. With this method the aldolase content of various normal rat tissues has been determined.

The authors are grateful to Dr. Sidney F. Velick of the Department of Biological Chemistry, Washington University, for a gift of crystalline aldolase, essentially free of isomerase.

*Addendum*—Since this manuscript was prepared, Professor C. F. Cori has informed us that the pH optimum for aldolase in the presence of carbonyl reagents is in part a reflection of the pH of optimum rate of combination of such reagents with triose phosphate. We have found (see the text) that the rate of aldolase action at pH

8.6 is independent of the presence of hydrazine when crystalline aldolase is used. However, the nature of the carbonyl reagent used also appears to have some effect on the reaction rate; cyanide definitely allows a higher rate than hydrazine. Due to these uncertainties about the optimum pH and whatever effects hydrazine may have on it and the activity of the enzyme, it is possible that the values we have given for different rat tissues may not be correct on an absolute basis. However, under the conditions used they are correct relative to each other, since alkali-labile P liberation and liberation of the triose chromogen are exactly parallel.

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# A PROCEDURE FOR THE DETERMINATION OF 4-AMINOSALICYLIC ACID (*p*-AMINOSALICYLIC ACID) IN BLOOD AND IN URINE\*

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(Received for publication, October 1, 1948)

At the present time there is considerable interest in 4-aminosalicylic acid (*p*-aminosalicylic acid) as a possible chemotherapeutic agent for use in tuberculosis, and a number of reports of *in vitro* and *in vivo* studies of this drug have appeared in the literature during the last few years. The concentrations of drug in blood and in urine following dosage with 4-aminosalicylic acid were first reported by Lehmann (1), who used Ehrlich's *p*-dimethylaminobenzaldehyde reagent for his determinations. This method suffers from lack of specificity, which makes interpretation of the results difficult, especially when the values are low. More recently Way, Smith, Howie, Weiss, and Swanson (2) have reported the results of analyses carried out by a modification of Bratton and Marshall's procedure (3) for the determination of sulfonamides; and Venkataraman, Venkataraman, and Lewis (4) have used ferric nitrate for the study of metabolic products of 4-aminosalicylic acid.

The analytical procedure described in the present paper is free from interference by the concentrations of unrelated substances normally occurring in blood and urine. It is based on the fact that 4-aminosalicylic acid will form either a red or a blue color under reaction conditions which differ only in the order in which the reagents are added. The colors formed by unrelated substances have the same intensities under both sets of conditions, and their interfering effect is eliminated by taking two aliquots of the unknown solutions, developing the red color with the drug in one, the blue color in the other, and subtracting their extinctions in the photoelectric colorimeter.

## Procedure

*Preparation of Samples*—Take 1 ml. of blood with 4 ml. of water, make up to 10 ml. with 95 per cent alcohol, mix, let stand 10 minutes, and centrifuge. Use 1 ml. of the supernatant for each aliquot in the analysis. Oxalate does not interfere.

Urine samples require no preparation other than dilution to a 4-aminosalicylic acid concentration of 0.25 to 5  $\gamma$  per ml.

\* Presented in part before the American Society of Biological Chemists at Atlantic City, March, 1948.

*Reagents*—Pyridine; sodium nitrite, 2 per cent; *p*-nitroaniline, 0.02 per cent; concentrated HCl; sodium hydroxide, 5 N.

The pyridine must be refluxed for several hours with potassium permanganate and distilled to remove impurities which form color in the reaction. Collect the distillate in several fractions and test each by a reagent blank procedure.

*Color Development*—Make up the coupling reagent freshly for each set of analyses by mixing 1 volume of 2 per cent sodium nitrite, 8 volumes of 0.02 per cent *p*-nitroaniline, and 1 volume of concentrated HCl.

Deliver duplicate aliquots of the unknown solutions, containing between 1 and 20  $\gamma$  of 4-aminosalicylic acid, into test-tubes placed in a water bath at about 20°, and make the volumes up to 4 ml. with water. Add 1 ml. of pyridine to the first tube of each set and 1 ml. of coupling reagent to the second. Mix well and then add 1 ml. of coupling reagent to the first tube and 1 ml. of pyridine to the second. Mix again, wait 5 minutes, and add 0.5 ml. of 5 N sodium hydroxide to each tube, mixing well afterward.

Measure the extinctions after 20 minutes. Place the contents of the first tube, which should be red, in the colorimeter and set the galvanometer spot at 100 per cent transmission, using a wave-length of 620  $m\mu$ . Then put the contents of the second tube, which should be blue, in the instrument and note the reading.

In the procedure as outlined, the final volumes of the solutions are 6.5 ml. If the colorimeter requires greater volumes, larger amounts of all of the components of the reaction may be used, or the solutions may be diluted with water before the color intensities are measured.

*Standards*—Run a standard determination with each set of analyses using 4 ml. aliquots of a solution which contains 2.5  $\gamma$  of 4-aminosalicylic acid per ml. When kept in the refrigerator, the standard solution is stable for about 30 days.

*Calculation of Results*—The extinctions ( $-\log T$ ) are proportional to the amount of 4-aminosalicylic acid present within the limits of accuracy of the procedure, and the analytical results with blood filtrates and urine dilutions may be compared directly with measurements on aqueous standards.

#### *General Considerations*

Either of the color-forming reactions used in this procedure is suitable for measuring the amount of 4-aminosalicylic acid in pure solution, but when they are applied separately to urine dilutions or to blood filtrates which contain no drug, the colors formed by interfering substances present give rise to apparent drug values of 100 to 200  $\gamma$  per ml. in urine and of more than 500  $\gamma$  per ml. in blood.

However, these interfering substances, which apparently are able to

couple in only one manner, produce equal intensity of color under both sets of reaction conditions, and their interfering effect is eliminated in the analytical procedure by subtracting their extinctions. Thus, when seven samples of blood from man, dog, and rabbit, to which no 4-aminosalicylic acid had been added, were analyzed, the average extinction was  $+0.0011$  (99.8 per cent transmission), and the range  $+0.0066$  to  $-0.0086$  (98.8 to 102.0 per cent). Similarly with urine, the average extinction was  $-0.0033$  (100.8 per cent transmission), and the range  $+0.0044$  to  $-0.0086$  (99.0 to 102.0 per cent). Also, while the conditions are sufficiently different to produce two colors with 4-aminosalicylic acid, the reagent blank values are equal, and their effect is eliminated in the same manner. Thus the average extinction of the reagent blanks in fifteen experiments, when measured against each other, was  $+0.0013$  (99.7 per cent transmission), and the range  $+0.0088$  to  $-0.0086$  (98.0 to 102.0 per cent).

When 4-aminosalicylic acid is treated with nitrous acid and then coupled with diazotized *p*-nitroaniline in the presence of pyridine as a promoter, the blue color is formed. The coupling reagent, which is added in the analysis before the pyridine, contains nitrous acid in excess and if this is first destroyed with ammonium sulfamate the red color is produced. The red color is formed in the analytical procedure when pyridine is added before the coupling reagent. Under these conditions reaction with nitrous acid does not occur. The red color can also be formed in the presence of bicarbonate buffer, but the addition of pyridine here is a convenient means of adjusting the solution to a favorable pH for coupling, of minimizing the number of reagents used, and of making the two reaction mixtures similar in composition.

*Reagents and Reagent Concentrations*—*p*-Nitroaniline was chosen for use after preliminary examination of a limited number of amines. Neither sulfanilic acid nor *p*-nitroaniline-*o*-sulfonic acid was found to be suitable.

The concentrations of the components of the coupling reagent are greater than those which give maximum extinction with aqueous solutions of 4-aminosalicylic acid. It was found that blood and urine contain one or more substances which inhibit color development, presumably by competing with the drug for the coupling reagent, and that the inhibition could be avoided by increasing the reagent concentration. This is illustrated in Fig. 1. The reagent chosen for use gives the same extinction with aqueous 4-aminosalicylic acid as with fortified blood and urine samples.

The chief inhibiting substance is uric acid. Its concentration is too low to influence the results when 1 ml. of a 1:10 dilution of blood or a 1:100 dilution of urine is used for analysis. When greater amounts of urine are required, the uric acid can be removed with silver lactate (5), but this is not recommended as a routine procedure, since it may yield low results.

**Absorption Spectra**—The absorption spectra of the colors produced are presented in Fig. 2. All of the measurements reported in this paper have been made with the Coleman model 11 spectrophotometer. The absorption maximum of the blue color compared with its reagent blank is 590  $m\mu$ , and of the red color, 540  $m\mu$ . The maximum difference between the two colors is at 615  $m\mu$ .

**Time Intervals**—The preliminary reaction between the 4-aminosalicylic acid and the excess nitrous acid in the coupling reagent is quite rapid.

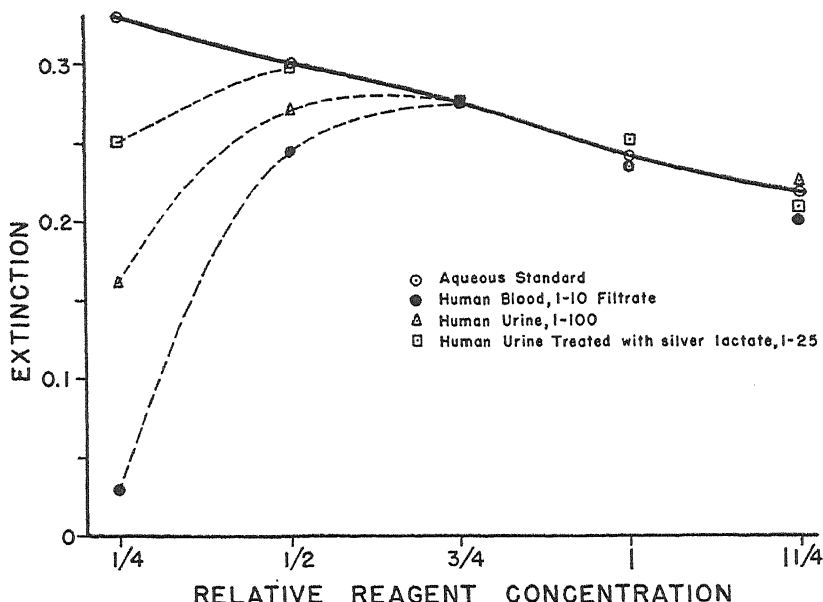


FIG. 1. Influence of variation of reagent concentration on the extinction produced by aqueous standards and biological samples. The concentration indicated at 1 is that used in the procedure.

There is no significant difference between analyses in which the pyridine is added 1 minute after the coupling reagent and those in which time intervals up to 6 minutes are allowed.

The influence of variation of the time interval between addition of pyridine and of sodium hydroxide is shown in Fig. 3. Here the final extinction is maximum when the coupling time is between 4 and 5 minutes.

The effect of variation of the time interval between addition of sodium hydroxide and measurement of the color intensities is shown in Fig. 4. Although there appears to be no significant variation here, the results are generally more consistent if the reactions are allowed to proceed for 20



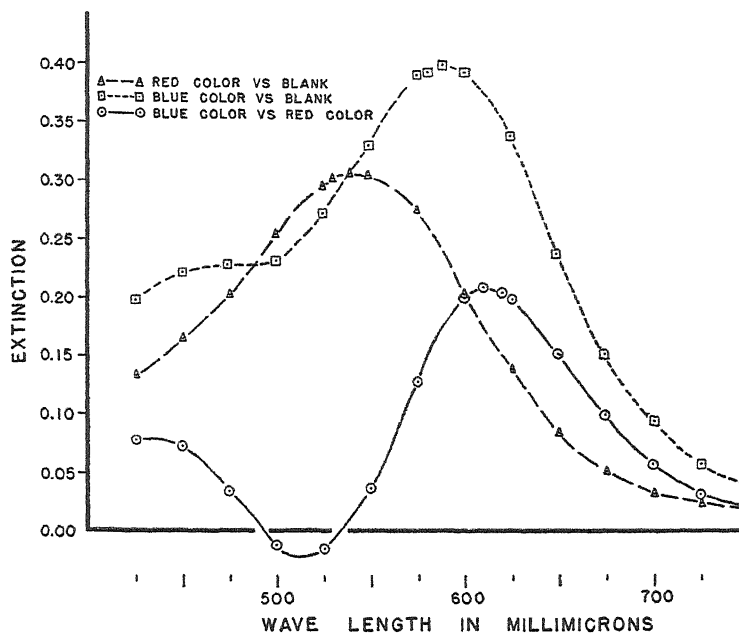


FIG. 2. Absorption spectra of the colors produced

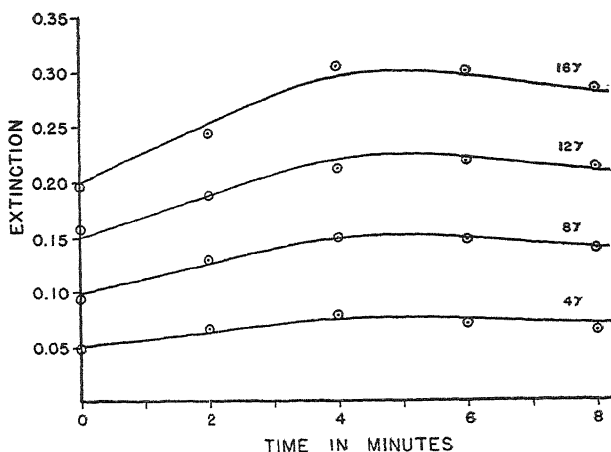


FIG. 3. The influence of variation of the time interval between the addition of pyridine and of sodium hydroxide on the final extinction. The transverse lines represent the indicated amount of 4-aminosalicylic acid.

minutes. The colors are quite stable for several hours. They do, however, deteriorate slowly over a period of 2 or 3 days.

*Temperature Control*—The reactions should be run at about 20° for best

agreement between aqueous standards and fortified biological samples. A series of experiments was run at 10°, 20°, and 30° to study the influence of temperature at the time of coupling on the final extinction of solutions

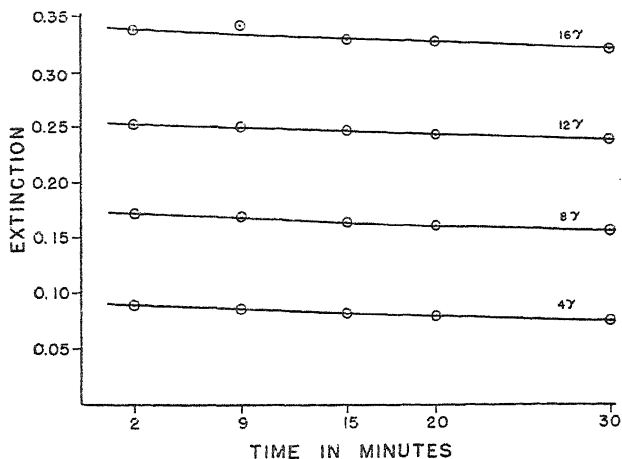


FIG. 4. The influence on extinction of time of standing before the color intensities are measured. The transverse lines represent the indicated amount of 4-aminosalicylic acid.

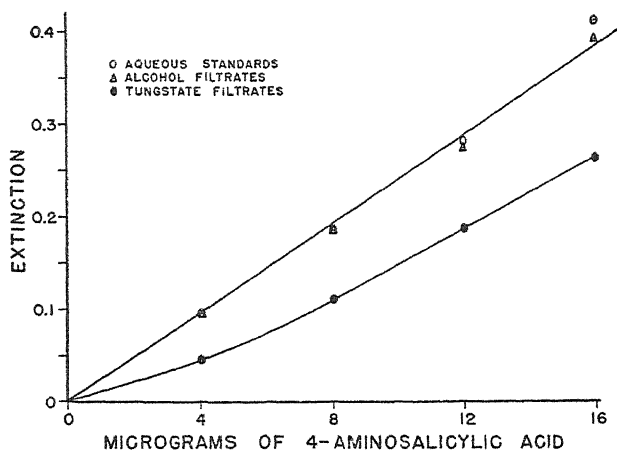


FIG. 5. A comparison of the recovery of 4-aminosalicylic acid from filtrates of fortified blood samples prepared with alcohol and with tungstate.

which varied in their 4-aminosalicylic acid content over the range of the assay procedure. In every case the extinction with aqueous standards was lowest at 30° and the extinction with filtrates prepared from fortified

blood was lowest at 10°, whereas at 20° the agreement between the two was quite satisfactory.

*Choice of Deproteinizing Agents*—Difficulties were encountered with all of the more common deproteinizing techniques, and alcohol has been used in this procedure since it was found to give reliable results. A comparison of the recovery of 4-aminosalicylic acid from fortified blood samples treated with alcohol and with tungstate is shown in Fig. 5. The analytical results with alcohol filtrates were essentially the same as those with untreated aqueous standards; but with tungstate filtrates the results were low, and

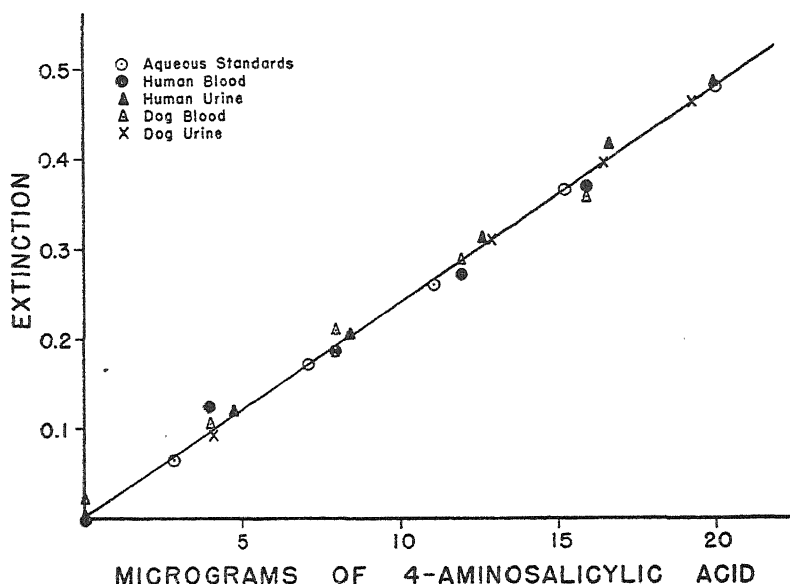


Fig. 6. Recoveries of 4-aminosalicylic acid from fortified samples of blood and urine from man and from dog.

the unrecovered 4-aminosalicylic acid could be extracted from the tungstate precipitate with alcohol.

*Recoveries from Fortified Samples*—When fortified samples of blood and urine from man and dog were analyzed, the results were in good agreement with those of aqueous standards, and the extinctions were proportional to the amount of 4-aminosalicylic acid present, as shown in Fig. 6. Statistical evaluation<sup>1</sup> of experimental data comparing fortified urine with aqueous standards showed that the variance of the difference between means of the extinction per microgram was one-tenth of the error variance. With

<sup>1</sup> The authors are indebted to Dr. Walther H. Ott for the statistical evaluations.

fortified blood samples the variance between means was one-half the error variance. The standard deviations were respectively 6 per cent and 8 per cent of the mean values.

*Interfering Substances*—The procedure as outlined is free from interference by normal concentrations of substances usually found in blood and in urine. This is illustrated by the data in Table I, which also includes the

TABLE I

*Extinction Produced by Given Amounts of Various Substances, Expressed As Apparent 4-Aminosalicylic Acid, and Inhibiting Effect of These Amounts on Extinction Produced by 10  $\gamma$  Samples of 4-Aminosalicylic Acid*

Substance	Amount	Apparent 4-aminosalicylic acid	Inhibition
	$\gamma$	$\gamma$	per cent
Allantoin .....	1000	0	0
Bilirubin .....	100	+3.7	-2.5
Creatinine .....	1000	0	0
Hippuric acid .....	1000	0	0
Urea .....	1000	0	0
Uric acid .....	30	0	45
Sodium salicylate .....	1000	0	0
Acetylsalicylic acid .....	1000	0	0
Penicillin G .....	1000	-1.1	0
Streptomycin .....	1000	0	0
Sulfanilamide .....	1000	-4.1	49
Sulfadiazine .....	1000	-6.7	87
Sulfapyridine .....	1000	-1.6	83
Sulfathiazole .....	50	-3.3	11
<i>m</i> -Aminophenol .....	10	+11.4	0
<i>p</i> -Aminobenzoic acid .....	20	-2.6	0
Anthranilic acid .....	20	-2.3	0
<i>p</i> -Aminohippuric acid .....	100	-2.5	0
Phenol .....	1000	0	82
Resorcinol .....	2	-5.2	0
Phloroglucinol .....	2	-1.6	11

influence of several commonly used drugs and of a few other compounds structurally related to 4-aminosalicylic acid.

Bilirubin produces green colors in the coupling reaction, resulting in a positive error which would be insignificant even at the concentrations present in cases of jaundice. Uric acid, however, when present in abnormally high amounts, will cause low analytical results, because it suppresses color formation on the part of 4-aminosalicylic acid.

Salicylates, penicillin, and streptomycin do not interfere with the analysis, but high concentrations of sulfonamides may cause low results. Of

the structurally similar compounds studied, *m*-aminophenol reacts in the same manner as 4-aminosalicylic acid, and produces colors of about the same intensity. Considerable amounts of color are also formed with resorcinol and phloroglucinol, but not with phenol.

#### SUMMARY

A colorimetric procedure for the determination of microgram amounts of 4-aminosalicylic acid in blood and urine has been described. Interference due to unrelated substances in biological samples has been eliminated by developing two colors with the drug and reading them against each other in the photoelectric colorimeter. When fortified samples of blood and urine were analyzed, the standard deviations of the results were respectively 8 and 6 per cent.

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# ALDEHYDE DEHYDROGENASE, A DIPHOSPHOPYRIDINE NUCLEOTIDE-LINKED ENZYME\*

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(Received for publication, October 7, 1948)

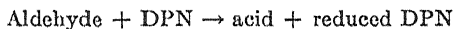
The dismutation of acetaldehyde to ethyl alcohol and acetic acid has been shown to occur in animal tissues (1, 2). The dependence of this reaction on diphosphopyridine nucleotide was demonstrated by von Euler and Brunius (3). Partial purification of the responsible enzyme system was achieved by Dixon and Lutwak-Mann (4). These authors clearly demonstrated that the mutase activity was independent of aldehyde (xanthine) oxidase activity and proposed that aldehyde mutase should be considered as a separate enzyme. This view has been accepted by recent text-books of enzymology in which the mutases are classified as separate enzymes (5, 6).

It is the purpose of this paper to show that the dismutation of aldehydes in the liver tissue is catalyzed by two separable enzymes. One is the known alcohol dehydrogenase recently obtained in crystalline form from liver tissue (7). The other is an aldehyde dehydrogenase which will be described in this paper. Both of these enzymes have been found to be present in considerable quantities in aldehyde mutase preparations purified according to Dixon and Lutwak-Mann (4).

## EXPERIMENTAL

### *Methods*

*Spectrophotometric Test for Determination of Aldehyde Dehydrogenase Activity*—The method is based on the reduction of diphosphopyridine nucleotide (DPN). This process, which is measured by the increase of absorption at 340 mμ, is catalyzed by the enzyme according to the following reaction:



In the presence of an excess of aldehyde and DPN the rate of reduction of DPN is a function of enzyme concentration (see Fig. 1) and can be used to measure enzymatic activity.

A Beckman quartz spectrophotometer was used for activity measurements. The solutions were pipetted into quartz cells. The control cell

\* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

contained all the solutions with the exception of the substrate. The determination was made at pH 9.3, which is the optimal pH for the aldehyde enzyme and also serves to reduce interference by oxidation of reduced DPN by other enzymes such as alcohol dehydrogenase.

The test was carried out in a final volume of 3 ml. in 0.01 M pyrophosphate buffer. Because of the kinetics of the reaction, which will be discussed later, the enzyme can be measured with the use of relatively small quantities of DPN and substrates. For routine testing of the enzyme activity, 200  $\gamma$  of DPN (65 per cent purity) and 500  $\gamma$  of acetaldehyde were used.

*Quantitative Determination of Acetic Acid*—When acetaldehyde is oxidized to acetic acid by aldehyde dehydrogenase, diphosphopyridine nucleotide acts as the only hydrogen acceptor. DPN, which can be used

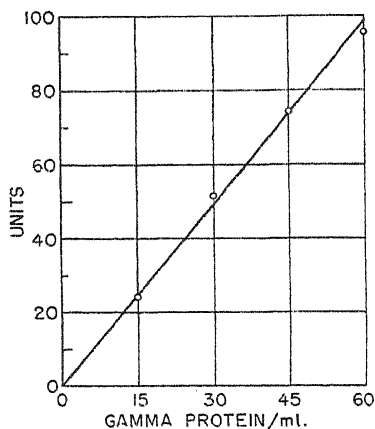


FIG. 1. Quantitative determination of aldehyde dehydrogenase. Relation of protein concentration to activity.

in catalytic amounts in the mutase system, must therefore be added in stoichiometric amounts for aldehyde oxidation in the absence of alcohol dehydrogenase. To determine the end-product of the reaction it was desirable to use a microtechnique.

Recently Hutchens and Kass<sup>1</sup> developed a quantitative colorimetric method for acetate determination based on the lanthanum color test. This method was found to be quite satisfactory with acetate solutions but could not be used in the presence of various impurities, as had already been pointed out by the authors.

It was possible to obtain reasonably good recoveries of acetate added to liver extracts by a combination of a micro diffusion technique with the colorimetric method of Hutchens and Kass.<sup>1</sup> The procedure of the micro

<sup>1</sup> Hutchens, J. O., and Kass, B. M., personal communication.



diffusion technique was based on the method of Black (8) but had to be modified in several respect for the above purpose. Deproteinization and evaporation of the sample to dryness were omitted and mercuric oxide and magnesium sulfate had to be added in order to obtain quantitative recoveries. The details of the test will be described later.

*Protein Determination*—Protein concentrations were determined spectrophotometrically at 280  $\mu$ . Corrections for nucleic acid impurities were made according to Warburg and Christian (9).

### *Materials*

DPN of 65 per cent enzymatic purity was prepared from bakers' yeast by the method of Williamson and Green (10) as modified by Ochoa.<sup>2</sup> TPN of 45 per cent purity was kindly supplied by Dr. S. Ochoa.

Glycolaldehyde was synthesized (11) and crystallized from the distillate (12). The other aldehydes used were commercially obtained preparations.

Alcohol dehydrogenase was crystallized from yeast according to Negelein and Wulff (13).

### *Results*

*Purification of Aldehyde Dehydrogenase*—Frozen beef liver, kept in a dry ice box, was used as starting material. An acetone-dried powder was prepared as follows: The thawed liver was mixed with 2 to 3 volumes of ice-cold acetone in a Waring blender, and the mixture poured into 8 volumes of acetone and then rapidly centrifuged in the cold room. The precipitate was washed once with 8 volumes of acetone, then pressed out between paper towels and crumbled into a fine powder which dried rapidly when distributed on large filter papers.

This powder was extracted with 8 volumes of distilled water at room temperature for 45 minutes under continuous mechanical stirring. From this point on all operations were carried out in the cold room. The mixture was centrifuged, and to each 100 ml. of the supernatant 70 ml. of cold 95 per cent ethyl alcohol were added in the course of 10 minutes. The temperature was allowed to rise to 12–14° during this procedure. After standing for an additional 20 minutes, the preparation was centrifuged, and to the clear supernatant 40 ml. of cold ethyl alcohol for each 100 ml. of the original extract were added slowly. After standing for 10 minutes at 0°, the precipitate was centrifuged off and dissolved in distilled water. The solution was dialyzed for 2 hours with mechanical stirring against large volumes of distilled water. The resulting precipitate was centrifuged off and discarded. For each gm. of protein present in the supernatant, 2 ml. of a neutralized 5 per cent solution of nucleic acid

<sup>2</sup> Ochoa, S., personal communication.

(Merck) were added, and the mixture was carefully adjusted to pH 5.2 with 0.1 M acetic acid. The precipitate formed was discarded even though considerable quantities of the aldehyde enzyme are present in this fraction. To the clear supernatant, half the amount of nucleic acid used in the above step was added, the pH readjusted to 5.2, and the mixture centrifuged. The precipitate was dissolved in dilute alkali and protamine sulfate (Squibb) was added in small fractions at pH 6.5 until the absorption ratio of 280  $m\mu$ :260  $m\mu$  indicated the removal of nucleic acid (9).

This preparation can be purified further by ammonium sulfate fractionation. However, most experiments were carried out with preparations purified to the extent described above, since no detectable alcohol dehydrogenase was found in this fraction.

The degree of purification obtained by this method was usually from 20- to 30-fold, with a yield of 20 to 40 per cent. A typical protocol of

TABLE I  
*Purification of Aldehyde Dehydrogenase from Beef Liver Acetone Powder*

Fraction	Volume of solution	Units*	Protein	Specific activity	Yield
	ml.		mg.	units per mg. protein	per cent
Aqueous extract.....	300	360,000	12,000	30	100
2nd alcohol ppt.....	100	300,000	2,000	150	83
2nd nucleic acid ppt. (after nucleic acid removal).....	10	140,000	200	700	38

\* A unit has been defined as the amount of enzyme activity causing an increase of optical density of 0.001 per minute.

purification and yields achieved is given in Table I. The enzyme thus purified is free of alcohol dehydrogenase and xanthine oxidase activity.

*Dismutation Reaction of Acetaldehyde*—As can be seen from Fig. 2, oxidation of acetaldehyde to acetic acid by the aldehyde dehydrogenase thus purified goes to completion in the absence of alcohol dehydrogenase. In the presence of alcohol dehydrogenase and an excess of acetaldehyde, the reduced DPN is reoxidized with simultaneous reduction of acetaldehyde to ethyl alcohol (Fig. 2). The result of the over-all reaction is a dismutation of acetaldehyde to acetic acid and alcohol, while DPN acting as a coenzyme to both dehydrogenases undergoes oxidation and reduction continuously. Considerable amounts of both dehydrogenases were found to be present in the preparations of mutase prepared according to Dixon and Lutwak-Mann (4).

All previous studies on the properties of the "mutase enzyme" were re-examined and interpreted in the light of the above findings. The data on

pH optimum, heat stability, and substrate specificity of the aldehyde enzyme were found to be at variance with those reported on mutase

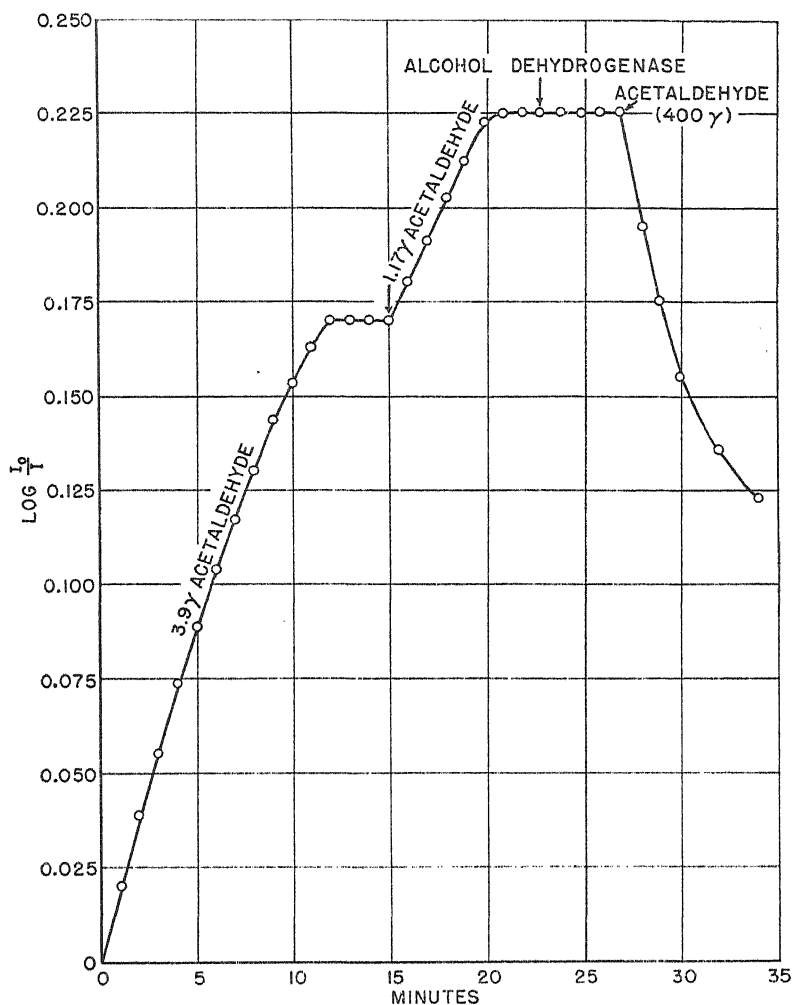


FIG. 2. Dismutation of acetaldehyde by combination of aldehyde dehydrogenase and alcohol dehydrogenase. DPN 0.0005 M, sodium pyrophosphate 0.01 M, pH 9.3.

activity but the discrepancies are readily accounted for by the participation of alcohol dehydrogenase in the activity of the "mutase enzyme."

*Coenzyme and Substrate Specificity*—Dixon and Lutwak-Mann (4) have demonstrated the specificity of coenzyme I (DPN) for mutase activity. Triphosphopyridine nucleotide (TPN) was inactive. This

specificity of DPN was fully confirmed for the aldehyde dehydrogenase. This is of particular interest in the light of recent findings of Ochoa and his collaborators (14), indicating that some of the dehydrogenases previously believed to be specific for DPN also react with TPN, although at a slower rate. In its specificity for DPN the aldehyde dehydrogenase is similar to glyceraldehyde phosphate dehydrogenase (14).

A number of aldehydes tested were found to be readily oxidized by the aldehyde dehydrogenase. These were formaldehyde, acetaldehyde, glycolaldehyde, propionaldehyde, butylaldehyde, isovaleraldehyde. The rate of formaldehyde oxidation, which was the least active of these substrates, was approximately half the rate at which the most rapidly attacked acetaldehyde was oxidized. Of special interest is the oxidation of salicylaldehyde, since this substance was found to be inactive when tested with the mutase preparation by Dixon and Lutwak-Mann (4). In fact, these authors listed the inability of the mutase preparation to oxidize aromatic aldehydes as one of the principal characteristics of mutase, differentiating it from aldehyde (xanthine) oxidase, which reacts with aromatic aldehydes as well. With the use of the spectrophotometric method it was possible to show that in the case of salicylaldehyde the difference in the substrate specificity is not due to the failure on the part of the oxidizing enzyme but rather due to the sluggish rate at which the reducing enzyme (alcohol dehydrogenase) attacks the substrate.

Benzaldehyde was also tested and was found to be inactive as a substrate. However, some interaction of this aldehyde with aldehyde dehydrogenase was suggested by a very marked inhibition of acetaldehyde oxidation in the presence of benzaldehyde. Purines which are oxidized by aldehyde (xanthine) oxidase are not attacked by the liver aldehyde dehydrogenase.

*Stability, pH Optimum, and Substrate Affinity*—After the purification outlined above, the enzyme becomes unstable and loses activity quite rapidly. In order to preserve it for analytical purposes (the quantitative determination of aldehydes), it was most convenient to store it in 0.02 M pyrophosphate buffer at pH 8.0, well stoppered and frozen in a dry ice box. A preparation thus stored had retained approximately 30 per cent of its original activity after 8 months. Stability is also improved if it is kept in 50 per cent ammonium sulfate at pH 8.0.

The aldehyde dehydrogenase enzyme is quite heat-labile. Heating to temperatures above 55° causes rapid loss of activity. Exposure to a temperature of 58° for 2 minutes results in 80 per cent inactivation.

The enzyme tested in 0.01 M pyrophosphate buffer shows a sharp optimum around pH 9.3 (see Fig. 3) with acetaldehyde as substrate.

From Fig. 2 it can be seen that the enzyme has a very high affinity for the substrate. In fact, it was impossible to determine accurately the

Michaelis constant under these experimental conditions, since  $K_m$  is less than  $10^{-5}$  M, a concentration below which absorption changes of reduced DPN are too small to be read with accuracy. This high affinity for the substrate makes the enzyme a suitable tool for the microdetermination of aldehydes. 0.03 to 0.5 micromole can be measured accurately by this method (see Fig. 2). Due to the reactivity of the enzyme toward a number of aldehydes the method lacks specificity.

*Identification of Oxidation Product*—For the identification of the oxidation product it was necessary to use stoichiometric amounts of DPN, since no alcohol dehydrogenase is present in the purified enzyme preparation.

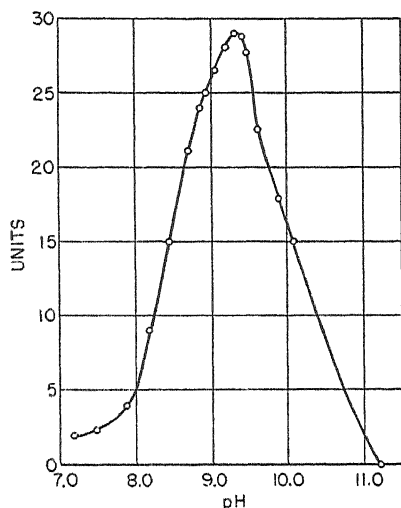


FIG. 3. Effect of pH on oxidation of acetaldehyde by aldehyde dehydrogenase in 0.01 M pyrophosphate buffer.

A typical experiment was set up as follows: 0.5 ml. of purified enzyme, 0.3 ml. of 0.1 M pyrophosphate buffer, 15 micromoles of enzymatically active DPN, and 10 micromoles of acetaldehyde were present in a final volume of 3 ml. By the addition of small amounts of 0.2 N sodium hydroxide, to neutralize the acetic acid formed, the mixture was kept at a pH of approximately 9.0, measured with the glass electrode. At 20 minute intervals, 0.03 ml. samples were withdrawn and the formed  $\text{DPNH}_2$  measured by the increased absorption at  $340\text{ m}\mu$ . In the above experiment close to 10 micromoles of reduced DPN (calculated according to Ohlmeyer's (15) molecular absorption coefficient) were found after 40 minutes, and additional incubation resulted in no further increase in absorption at  $340$

mμ. The entire sample was transferred to a screw-capped glass bottle, 4 cm. in diameter and about 5 cm. high. 2 gm. of magnesium sulfate and approximately 500 mg. of mercuric oxide were added. A center cup of about 2 cm. in diameter and 1 cm. in height was then placed in the bottle on a support of glass raised well above the fluid level. Into the center cup was pipetted 0.11 ml. of a 15 per cent potassium iodide solution in 0.1 N sodium hydroxide. Finally, 1 ml. of 15 N  $\text{H}_2\text{SO}_4$  was rapidly pipetted into the main compartment of the bottle, any spilling on the center cup being avoided. The opening of the bottle was quickly covered with aluminum foil and the cap tightly screwed on. The samples were then placed in an air oven at 105° for 24 hours.

After this period the center cup was washed with small amounts of water, the washings pooled, and the acetate determined on an aliquot containing about 3 micromoles of acetic acid. Known amounts of acetic acid between 80 to 200 γ, neutralized with the potassium iodide-sodium hydroxide solution, were run at the same time.

The amount of alkali used in the center cup in the above experiment was adjusted to an amount only in slight excess of that necessary to neutralize the expected acid, since a larger excess of alkali interferes with the colorimetric test. In case of unknown quantities of acetic acid, the excess alkali can be neutralized with nitric acid to pH 8.2, with phenolphthalein as external indicator.

Control vessels, either without DPN or with boiled enzyme solutions, were run simultaneously with the samples containing all active components. Recovery experiments in the presence of all reagents were performed by adding 10 micromoles of acetic acid instead of acetaldehyde.

In the above experiment 9.3 micromoles of acetic acid formed from acetaldehyde were obtained. No acetic acid was found in a control experiment in which DPN was omitted. In another control in which 10 micromoles of acetic acid were added instead of acetaldehyde, 10.3 micromoles of acetic acid were recovered.

*Attempts to Test Reversibility of Reaction*—With the end-product thus established as acetic acid, attempts were made to test the reversibility of this reaction. Sodium acetate with reduced DPN failed to react with the enzyme. Likewise, a reversal of the reaction was not obtained with acetyl phosphate or with acetate and adenosine triphosphate. The latter experiments were performed with active purified preparations of aldehyde dehydrogenase with and without addition of a crude liver extract or of a highly purified preparation of the enzyme catalyzing the phosphorylation of 3-phosphoglyceric acid (16).

These experiments were carried out because of certain similarities between the above reaction and the oxidation of glyceraldehyde phosphate

by triose phosphate dehydrogenase. In addition, recently Stadtman and Barker (17) showed a participation of phosphate in the oxidation of acetaldehyde by *Clostridium kluyveri*. However, no evidence was found for the participation of phosphate in the oxidation of acetaldehyde by the liver dehydrogenase. The reaction catalyzed by purified, well dialyzed preparations proceeds in the absence of added phosphates.

#### DISCUSSION

A conclusive demonstration of the existence of a single enzyme catalyzing a Cannizzaro reaction is not available. The evidence presented in this paper reveals that aldehyde mutase, the last of the enzymes still classified as mutases in text-books, consists of two separable enzymes, at least in the case of liver preparations.

Differences in the properties of the aldehyde dehydrogenase and the "mutase" are readily explained by the participation of alcohol dehydrogenase in the dismutation reaction. The absence of dismutation of some aromatic aldehydes is due to the sluggish reaction of the reducing enzyme with this substrate. The rate of oxidation of salicylaldehyde is of the same order as that of aliphatic aldehydes.

The reported absence of alcohol dehydrogenase in the mutase preparation of Dixon and Lutwak-Mann (4) can be explained by the accidental removal during the purification process of diaphorase, which is required in their test system. This possibility was later suggested by Dixon (18).

#### SUMMARY

1. Purified aldehyde mutase preparations contain two separable enzymes, alcohol dehydrogenase and aldehyde dehydrogenase, which together catalyze the dismutation of aldehydes.

2. The purification of a diphosphopyridine nucleotide-linked aldehyde dehydrogenase from beef liver is reported.

3. The results presented in this paper suggest that mutases catalyzing the Cannizzaro reaction may not exist as single enzymes.

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## INTRACELLULAR DISTRIBUTION OF ENZYMES

### IV. THE DISTRIBUTION OF OXALACETIC OXIDASE ACTIVITY IN RAT LIVER AND RAT KIDNEY FRACTIONS\*

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(Received for publication, October 11, 1948)

The "oxalacetic acid oxidase system" has been defined as the complex of enzymes that are required for the oxidation of oxalacetic acid (8). It has been so named because oxalacetic acid is the substrate that has been employed and because it is realized that the oxidation is brought about by a "system" of enzymes rather than by a single catalyst. Although the oxidation probably proceeds via the Krebs cycle of oxidations ((9) and unpublished work<sup>1</sup>) and the properties of the system are essentially the same as those of "cyclophorase" (1), we do not obtain identical results with all of the members of the Krebs cycle (9) and hence for the present prefer to retain the name that is descriptive of the initial substrate employed, while recognizing that other members of the cycle may be quickly formed.<sup>1</sup> The oxidation is coupled with the phosphorylation of the adenosine triphosphate (ATP) system and has been demonstrated in liver, kidney, brain, heart, and skeletal muscle, but not in tumor homogenates (8).

Since methods have been developed for the separation of rat tissue homogenates into cytologically distinct fractions (3, 13) and since the oxalacetic oxidase system would appear to involve most if not all of the enzymes of the Krebs cycle, it was of interest to determine whether the oxalacetic oxidase activity of rat tissue homogenates was associated exclusively with a single component of the cell or whether several components were necessary for complete activity. Such a study would also help to decide the question of whether the system is bound together into one particle or whether the several activities are separable. Although the latter situation was actually observed, it should be emphasized that the results do not necessarily conflict with the concept that is implied in the term "cyclophorase" that has been employed by Green *et al.* (1), since there may well be a particulate unit with the bulk of the enzymes

\* This work was aided by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

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<sup>1</sup> Potter, V. R., and Lyle, G. G., unpublished work.

of the Krebs cycle incorporated into it, with supplementary activities carried on other particles or in solution (*cf.* Potter and Albaum (6)). On the other hand, it seems likely that the centrifugal technique that has been used for the preparation of "cyclophorase" (1) would yield a mixture of several cell components, and although the techniques for the separation of such components (3, 13) are not yet fully perfected, they do make it possible to obtain various fairly well defined fractions, and to test their activities relative to each other and to the original whole homogenate.

In the present study, rat liver and kidney homogenates were separated by differential centrifugation into fractions containing nuclei, mitochondria, submicroscopic particles, and supernatant or "soluble" proteins. The oxalacetic oxidase activity of the single and combined fractions was then compared with that of the whole homogenates. In this work the mitochondria were obtained in one well defined fraction that was free from nuclei and probably free of soluble enzymes and submicroscopic particles (3); the latter two fractions were free from mitochondria or other particles that could be resolved in the light microscope. It should be emphasized, however, that there is no implication that the mitochondrial fraction consists of identical particles; it is quite likely that there are many varieties of mitochondria not only in different tissues but in any given cell.

#### EXPERIMENTAL

##### *Materials and Methods*

*Preparation of Tissues*—The tissues were obtained from rats originally of the Sprague-Dawley strain obtained from the Holtzman Rat Company, Madison. In the case of the experiments with liver, the animals were fasted overnight to eliminate glycogen from the liver. The rats were killed by decapitation and the tissues were removed and chilled in ice-cold isotonic KCl. After cooling, the tissues were blotted, weighed, and homogenized (7) in 9 volumes of isotonic sucrose (8.5 gm. of sucrose per 100 ml. of solution).

*Differential Centrifugation of Tissue Homogenates*—The centrifugation of the tissue homogenates was carried out essentially as described in Paper III in this series (13). Four fractions were obtained: a nuclear fraction,  $N_w$ ; a mitochondrial fraction,  $M_w$ ; a submicroscopic particle fraction,  $P_w$ ; and a supernatant or "soluble" protein fraction,  $S_2$ . In addition to these fractions an aliquot of the extract,  $E$ , remaining after removal of the nuclear fraction was saved for analysis. As was pointed out previously (13), the nuclear fraction contained some mitochondria and whole cells in addition to nuclei. Thus this fraction was impure from the cytological standpoint, even though all the nuclei of the homogenate were

concentrated in this fraction. The mitochondrial fraction is apparently cytologically pure (3).

*Measurement of Oxalacetic Oxidase Activity*—Oxalacetic oxidase activity was measured in the Warburg apparatus with the optimum conditions that had been determined by Potter *et al.* (9). The flasks contained 0.0167 M potassium phosphate of pH 7.0, 0.067 M KCl, 0.0017 M  $MgCl_2$ , 0.0000133 M cytochrome *c*, 0.00167 M adenosine triphosphate (K salt), 0.00178 M oxalacetic acid, and 0.00267 M pyruvic acid. The oxalacetic and pyruvic acids were made up as a mixture to which 0.16 M  $K_2CO_3$  was added equivalent to three-fourths of the oxalacetic acid and all of the pyruvic acid. The flasks also contained 1.2 ml. of 8.5 per cent sucrose solution or its equivalent in the form of the homogenate or the tissue fractions. The final volume of the flask contents was 3.0 ml. Readings of oxygen uptake were made every 10 minutes after a temperature equilibration period of 6 to 8 minutes.

*Analytical Methods*—Desoxypentose (DNA) and pentose (PNA) nucleic acids were extracted with hot trichloroacetic acid and were estimated by means of colorimetric reactions (10). Total nitrogen was determined colorimetrically after acid digestion (15).

*Reagents*—Oxalacetic acid was prepared as described previously (15). Pyruvic acid (Eastman) was distilled under reduced pressure in an all-glass apparatus. Adenosine triphosphate (Ba salt) was generously supplied by Dr. G. A. LePage and was prepared as previously described (15). Cytochrome *c* was prepared by the method of Keilin and Hartree with minor modifications (15).

### Results

*Distribution of Nucleic Acids and Total Nitrogen*—The distribution of nucleic acids and total nitrogen in the rat liver and kidney fractions is given in Tables I and II. The entire DNA of the rat liver and kidney homogenates was recovered in the nuclear fractions obtained from these homogenates. This finding confirmed the microscopical examination of the fractions, which indicated that nuclei or nuclear fragments were absent from all but the nuclear fraction. The PNA:N ratio was found to be increased only in the submicroscopic particle fraction of rat liver, which also contained the largest proportion of the total PNA, as was observed in previous work (3). In the case of rat kidney, however, the PNA:N ratio was increased in the submicroscopic particle fraction only to a slight extent and the largest amount of PNA was recovered in the supernatant fraction. Thus the distribution of PNA appears to be considerably different in rat kidney than in rat liver. The distribution of total nitrogen was also considerably different in the kidney as com-

pared to the liver fractions. The nuclear fraction of the kidney contained considerably more nitrogen than the nuclear fraction of the rat liver, while the mitochondrial and submicroscopic particle fractions of the rat kidney contained substantially less nitrogen than did the corresponding fractions of rat liver.

*Distribution of Oxalacetic Oxidase Activity in Rat Liver Fractions*—The oxalacetic oxidase activity of rat liver fractions is given in Table I. The mitochondrial fraction was the most active fraction obtained and

TABLE I  
*Distribution of Nitrogen, Nucleic Acids, and Oxalacetic Oxidase Activity in Rat Liver Fractions*

Each figure represents the average of two experiments.\*

Tissue fraction	Total nitrogen†	PNA phosphorus‡	DNA phosphorus‡	Oxalacetic oxidase activity†	
				Total	Fraction of homogenate
	$\gamma$	$\gamma$	$\gamma$	<i>c.mm. O<sub>2</sub> per 10 min.</i>	<i>per cent</i>
Homogenate	2780	78.9	28.5	103	(100)
Nw	402	9.3	28.7	10.8	10.5
E	2210	70.1		93.1	90.5
Nw + E				126	122
Mw	700	8.2		45.8	44.5
Pw	504	36.3		0	0
S <sub>2</sub>	1098	26.2		5.2	5.0
Mw + Pw				84.0	81.6
" + S <sub>2</sub>				63.8	62.0
Pw + "				8.8	8.6
Mw + Pw + S <sub>2</sub>				101	98.1
Nw + Mw + Pw + S <sub>2</sub>				121	118

\* The figures for the oxygen uptake of the whole homogenates and the washed mitochondria (Mw) are well within the range observed in experiments in which the other fractions were not studied.

† Per 100 mg. of fresh liver or its equivalent.

‡ Phosphorus calculated from pentose determinations (10).

contained 44.5 per cent of the activity of the homogenate, as compared with 10.5, 5.0, and 0 per cent in the nuclear, submicroscopic particle, and supernatant fractions, respectively. The recombination of the fractions resulted in several synergistic or activating effects. The most pronounced effect was obtained when the submicroscopic particle fraction (which had no activity by itself) was added to the mitochondrial fraction. The addition of the supernatant fraction to the mitochondria had a similar but less striking effect. The recovery of enzymatic activity in the recombined fractions was considerably greater than expected. Thus the com-

binations ( $N_w + E$ ) and ( $N_w + M_w + P_w + S_2$ ) should each equal the activity of the homogenate, and the activity of the combination ( $M_w + P_w + S_2$ ) should equal the activity of the extract E. The activity of the former two combinations was, however, 122 and 118 per cent that of the homogenate, while the activity of the latter combination was 108 per cent of the extract. No adequate explanation can be provided for these increased activities.

TABLE II

*Distribution of Nitrogen, Nucleic Acids, and Oxalacetic Oxidase Activity in Rat Kidney Fractions*

Each figure represents the average of two experiments.\*

Tissue fraction	Total nitrogen†	PNA phosphorus‡	DNA phosphorus‡	Oxalacetic oxidase activity†	
				Total	Fraction of homogenate
	$\gamma$	$\gamma$	$\gamma$	<i>c.mm. O<sub>2</sub> per 10 min.</i>	<i>per cent</i>
Homogenate	2204	34.5	43.1	186	(100)
Nw	509	5.4	45.5	16.0	8.6
E	1785	31.9		138	74.2
Nw + E				167	89.8
Mw	435	4.2		45.5	24.4
Pw	351	8.4		0	0
S <sub>2</sub>	1031	19.8		0	0
Mw + Pw				18.5	10.0
" + S <sub>2</sub>				54.5	29.3
Pw + "				2.0	1.1
Mw + Pw + S <sub>2</sub>				10.0	5.4
Nw + Mw + Pw + S <sub>2</sub>				53.0	28.5

\* The figures for the oxygen uptake of the whole homogenates and the washed mitochondria (Mw) are well within the range observed in experiments in which the other fractions were not studied.

† Per 100 mg. of fresh whole kidney or its equivalent.

‡ Phosphorus calculated from pentose determinations (10).

*Distribution of Oxalacetic Oxidase Activity in Rat Kidney Fractions*—The distribution of oxalacetic oxidase activity in rat kidney fractions (Table II) was similar to the distribution of this enzyme complex in rat liver fractions. The highest activity was again associated with the mitochondria fraction but constituted only 24.4 per cent of the activity of the homogenate. The total activity of the kidney mitochondria was about the same as for those from liver, but their total nitrogen was less and therefore their activity on a nitrogen basis was necessarily higher than in the case of the liver. It should be noted that we are dealing with a fairly uniform cell type in the case of liver, while in kidney prepara-

tions many types of cells contribute to the various fractions. The recovery of oxalacetic oxidase activity in the kidney fractions was not complete. Thus the addition of the nuclear fraction to the extract ( $N_w + E$ ) resulted in an activity that was 89.8 per cent that of the homogenate. The addition of the submicroscopic particles to the mitochondria resulted in marked inhibition of the activity of the mitochondria and the recombination of all fractions ( $N_w + M_w + P_w + S_2$ ) resulted in an activity that was only 28.5 per cent that of the homogenate. These results were in striking contrast to those obtained with liver (Table I), in which the submicroscopic particles greatly increased the activity of the mitochondria and the activity of the recombined fractions exceeded that of the homogenate.

The failure to obtain complete recovery of enzymatic activity in the rat kidney fractions could be interpreted as a greater lability of one or several of the fractions in the isolated state. Potter, LePage, and Klug (8) had already observed that the oxalacetic oxidase activity of rat kidney homogenates was considerably less stable than that of liver homogenates. Thus it seemed reasonable that the isolated kidney fractions might be expected to be more labile than the liver fractions. This view was also supported by the fact that the oxalacetic oxidase activity obtained when the nuclear fraction and the extract were recombined (Table II) gave a reasonable recovery of activity (89.9 per cent), while the recombination of the *fractions obtained from the extract* with the nuclear fraction yielded a much lower activity (28.5 per cent). This hypothesis was tested further by the omission of the isolation of the submicroscopic particles from the fractionation procedure. This permitted the assay of the homogenate and fractions about 3 hours after the animal had been killed, in contrast to the 6 hours required when the complete fractionation was made. The results of these fractionations are given in Table III. The data show that the supernatant,  $S_1$ , greatly increased the activity of the mitochondria when the oxalacetic oxidase assays were made immediately after the completion of the fractionation (3 hours after the animal was killed). When the fractions were maintained at 0° for an additional 3.5 hours (corresponding to the time required for the fractionations in Table II), the supernatant had completely lost its ability to stimulate the mitochondria and in fact decreased the activity of the mitochondria (6.5 hours, Table III). It is of interest to note that the nuclear fraction also markedly increased the activity of the mitochondria. The nuclear fraction, however, retained its ability to increase the activity of the mitochondria upon storage in the cold. These results indicate that the distribution is basically the same as in liver; namely, that the supernatant contains a factor or factors that stimulate the oxalacetic oxidase activity of the mitochondria. This factor is, however, much more labile in the kidney. The factors present in the

supernatant and in the nuclear fractions may or may not be identical. If both fractions contain the same stimulating agent, it is apparent that the factor is considerably more stable in the nuclear fraction than in the supernatant. The results in Table III also lead to the conclusion that the mitochondria are the most complete with respect to the enzymes of the Krebs cycle, and that, if these enzymes can be said to be localized in any single type of cellular inclusion, it must be in the mitochondria. This conclusion is supported by the fact that the mitochondrial fraction possesses a higher oxalacetic oxidase activity than any other single fraction as well as by the fact that the recombination of the nuclear and supernatant fractions ( $N_w + S_1$ ) results in a very low activity. These

TABLE III  
*Oxalacetic Oxidase Activity of Rat Kidney Fractions*

Each figure represents the average of two experiments.

Tissue fraction	Oxalacetic oxidase activity*			
	3 hrs. post mortem		6.5 hrs. post mortem	
	Total	Fraction of homogenate	Total	Fraction of homogenate
	<i>c.mm. O<sub>2</sub> per 10 min.</i>	<i>per cent</i>	<i>c.mm. O<sub>2</sub> per 10 min.</i>	<i>per cent</i>
Homogenate	226	(100)	187	(100)
N <sub>w</sub>	18.0	8.0		
M <sub>w</sub>	73.0	32.3	65.0	34.8
S <sub>1</sub> †	2.2	1.0		
N <sub>w</sub> + M <sub>w</sub>	141	62.4	115	61.5
“ + S <sub>1</sub>	13.0	5.8		
M <sub>w</sub> + “	149	66.0	42	22.4
N <sub>w</sub> + M <sub>w</sub> + S <sub>1</sub>	193	85.5		

\* Per 100 mg. of fresh kidney or its equivalent.

† This fraction is equivalent to fractions P<sub>w</sub> and S<sub>2</sub>, Table II.

fractions apparently act only when mixed with the mitochondria and can therefore be considered only as accessory factors.

*Experiments with Cyclophorase*—In view of the foregoing results it seemed desirable to determine the relationship between these particles and those employed by Green *et al.* (1). A sample of KCl homogenate (Waring blender) from rabbit kidney together with an aliquot of the twice washed particle fraction (R<sub>3</sub>K) was prepared by Dr. Green and generously provided for our measurements. The R<sub>3</sub>K was made up in KCl to a volume equivalent to one-fourth the volume of blender homogenate from which it was derived, and which had contained 13.2 per cent fresh rabbit kidney. Each preparation was tested at levels corresponding to

26.4 and 52.8 mg. of fresh kidney per flask in the system employed in this paper, except that the ATP addition was varied from 0 ml. to 0.1 and 0.3 ml. of a 0.01 M solution (Table IV). Just as in the case of isolated mitochondria and nuclei, the oxygen uptake was considerably lower in the "cyclophorase" than in the original blender homogenate, although the activity was increased per unit of nitrogen or PNA. The oxygen uptake represented a higher fraction of the original than was obtained in the case of the mitochondrial fraction,  $M_w$ , and was more comparable to the values obtained with the combined nuclei and mitochondria, as shown in Table III. The analytical data also supported the conclusion that the cyclophorase preparation contained both nuclei and mitochondria, although not all of the DNA was accounted for. The PNA recovered in

TABLE IV

*Distribution of Nitrogen, Nucleic Acids, and Oxalacetic Oxidase Activity in Rabbit Kidney Blender Homogenate and in "Cyclophorase"*

Preparation	Total nitrogen*	PNA phosphorus*	DNA phosphorus*	Oxygen uptake for 40 min.		
				No ATP	0.1 ml. ATP†	0.3 ml. ATP†
				<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>
A. Blender homogenate	1710	27.6	44.0			
0.2 ml.				21	53	58
0.4 "				32	108	128
B. Cyclophorase	581	6.2	32.4			
0.2 ml. A				0	11	20
0.4 " "				0	41	53

\* Per 100 mg. of fresh kidney or its equivalent.

† Amount of 0.01 M ATP added per 3 ml. of flask contents.

the cyclophorase preparation was 6.2  $\gamma$  from 27.6  $\gamma$  in the blender homogenate as compared with 4.2  $\gamma$  from 34.5  $\gamma$  in the case of mitochondria and rat kidney homogenate or 9.6  $\gamma$  from the sum of nuclei and mitochondria (Table II).

#### DISCUSSION

Previous work has shown that several of the individual enzymes of the Krebs tricarboxylic acid cycle were associated with the mitochondria of various rat tissues. Thus the enzymes succinic dehydrogenase and cytochrome oxidase were found to be associated almost exclusively with the mitochondria or large granules isolated from rat liver, kidney, and liver tumors (2, 3, 11, 12). Cytochrome c was also found to be associated with rat liver mitochondria (14). In addition the enzyme complex



that oxidizes fatty acids to acetoacetic acid was also found to be associated almost exclusively with the mitochondria of rat liver (4, 13). In the case of the fatty acid oxidase it is now believed that the oxidation of the fatty acid results in the formation of 2-carbon fragments<sup>W</sup> that condense with each other to form acetoacetic acid or condense with oxalacetic acid and are metabolized to  $\text{CO}_2$  and water via the Krebs cycle (5). Thus a considerable body of evidence was available to indicate that the enzymes of the Krebs cycle and those closely associated with it are in part or exclusively associated with the mitochondrial fraction.

The data presented in this paper offer further support for this view, inasmuch as the mitochondrial fractions of rat liver and kidney homogenates had much higher oxalacetic oxidase activities than any of the other fractions. The addition of the other fractions to the mitochondrial fraction did, however, result in a greatly increased activity of the mitochondria. It is not clear whether the addition of the fractions to the mitochondria was actually an addition of some of the enzymes of the Krebs cycle or whether some accessory factor required by the cycle was being added.

The increased activity of the mitochondria that is obtained when nuclei are combined with them (Table III) is of considerable interest in connection with the results of Green *et al.* (1). These investigators obtained "cyclophorase" preparations by centrifuging and washing the particles that could be obtained from KCl homogenates by means of moderate gravitational forces (15 minutes at  $2000 \times g$ ). They referred to "enzymes constituting the cyclophorase complex" and commented on the nucleoprotein nature of the preparation. With the cooperation of Dr. Green it was possible to show that the cyclophorase preparation is comparable to a mixture of nuclei and mitochondria (*cf.* Tables III and IV) and that it contains both nuclear and cytoplasmic nucleoprotein. The results with the separated fractions showed that, when mitochondria having 32.3 per cent of the homogenate activity were mixed with nuclei having 8.0 per cent, the mixture showed 62.4 per cent of the homogenate activity (Table III). Since the mitochondria were devoid of DNA, there seems to be no reason to believe that the oxidation is dependent upon it, although the contribution of the nuclei to the oxygen uptake shown by mixtures of mitochondria and nuclei is not clear. The rôle of the PNA in the mitochondria also remains to be seen.

In Paper I of this series, the necessity for determining whether the sum of the activities of the tissue fractions was equal to the activity of the original homogenate was emphasized (11). Such a result could be obtained in the case of the succinoxidase system and indicated that the same catalyst was limiting in every fraction and in the whole homogen-

ate. In the present work, the sum of the activities of the separate fractions did not equal the activity of the whole homogenate, and it must therefore be concluded that the activity observed in the whole homogenate depends upon several enzyme activities and that the rate-determining enzyme is not the same in the different fractions. In this work it was found that several fractions with low activity alone or in mixtures produced considerable increases in activity when added to the mitochondrial fraction. Measurements of activity in the mitochondria alone, without reference to the whole homogenate or to the mixtures of mitochondria and other fractions, would have had little significance as to the rôle of the mitochondria in this system. The available data leave many questions unanswered, but there are strong indications that the mitochondria represent the major site of the Krebs cycle oxidation in tissues examined. Further studies will be required to determine what activities are in the other fractions and how these activities affect the activity of the mitochondria.

Finally it may be noted that for assay purposes, in which it is desired to compare one tissue with another or a normal tissue with an abnormal tissue, the whole homogenate should always be tested. Only when a fraction can be obtained that surpasses the activity of the whole homogenate will it be appropriate to consider the use of a derived fraction for assay purposes, or to assume the presence of inhibitors in the whole homogenate. By separating the component enzyme activities of a complex such as the oxalacetic oxidase system, the centrifugal fractionation procedure may be able to shed light on the nature of the enzyme systems in addition to contributing to the cytochemical aspects. However, in a system in which the rate-determining enzyme cannot exhibit activity without the coordinated function of a number of other enzymes that are in different cell fractions, the whole homogenate will give the greatest activity and the other fractions cannot be assayed with finality until they can be tested in the presence of purified preparations of all of the other members of the enzyme system. The centrifugal technique offers an excellent starting point for the preparation of some of these components.

#### SUMMARY

1. Rat liver and kidney homogenates were fractionated by differential centrifugation into nuclear, mitochondrial, submicroscopic particle, and "soluble" protein fractions and the distribution of nucleic acids and oxalacetic oxidase in these fractions was determined.

2. The distribution of the nucleic acids in these fractions was similar to that described in previous work. Desoxypentose nucleic acid was recovered entirely in the nuclear fractions, while pentose nucleic acid (PNA) was found in all the fractions.

3. The mitochondrial fractions of these tissues had the highest oxalacetic oxidase activity of the fractions obtained, but this activity accounted for only about 45 and 30 per cent of the activity of the liver and kidney homogenates respectively. The activity of the other fractions was much lower than that of the mitochondria, and the recovery of oxalacetic oxidase activity in the separate fractions was incomplete but approximately complete recovery was obtained when the enzymatic activities were determined on the recombined fractions.

4. The significance of these findings was discussed. It was concluded that the major portion of the enzymes of the oxalacetic oxidase system is probably associated with the mitochondria of rat liver and kidney, but that the full activity of these enzymes is limited by the presence in the other fractions of accessory enzymes or coenzymes.

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# UNIDENTIFIED FACTORS REQUIRED BY LACTOBACILLUS CASEI\*

## III. THE NATURE OF STREPOGENIN ACTIVITY†

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(Received for publication, September 15, 1948)

Woolley (3) reported the presence of a growth factor in the aqueous liver extract which is insoluble in 70 per cent ethanol (Wilson's liver fraction L) essential for certain hemolytic streptococci on a casein hydrolysate basal medium containing glutamine. Sprince and Woolley (4) later found this factor, which they termed "strepogenin," to be required by *Lactobacillus casei* and *Streptococcus lactis* when the casein hydrolysate medium of Landy and Dicken (5) containing no glutamine was used. Woolley (6) has reported glutamine, however, to be 50 to 200 times as active as Wilson's liver fraction L in strepogenin potency for *Lactobacillus casei* when added to the casein hydrolysate basal medium.

Pollack and Lindner (7) and Chu and Williams (8) found glutamine capable of replacing an unidentified factor in Wilson's peptone for *Lactobacillus casei*. Wright and Skeggs (9), Totter and King (10), and Woolley (6) have reported work which has implicated glutamic acid and glutamine metabolism and strepogenin activity.

Scott, Norris, and Heuser (1) and Daniel, Scott, Heuser, and Norris (2) have presented evidence that *Lactobacillus casei* requires for maximum growth a factor, or factors, associated with animal products in addition to strepogenin. The unadsorbable activity in trypsin-digested casein, which is not precipitated by lead, was considered to be strepogenin.

In view of these reports, studies were undertaken to obtain a better conception of the nature of strepogenin activity, and to find a pure source of this activity which would be suitable for supplementing a basal medium for the microbiological assay of unidentified chick growth factors.

\* This work was undertaken in cooperation with the Office of Naval Research, Navy Department, Washington, D. C., and the Nutrition Foundation, Inc., New York, and was aided by grants to Cornell University by the Cerophyl Laboratories, Inc., Kansas City, Missouri, and the Western Condensing Company, San Francisco, California. This work was conducted in the Nutrition Laboratories of the Department of Poultry Husbandry.

† The publications by Scott, Norris, and Heuser (1) and Daniel, Scott, Heuser, and Norris (2) constitute the first two papers in this series.

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## EXPERIMENTAL

The following studies were conducted with a culture of *Lactobacillus casei* 7469 obtained from the American Type Culture Collection. The methods of maintaining it and the procedures for inoculation and incubation were the same as those described by Daniel, Scott, Heuser, and Norris (2).

*Media*—Two media were used in the experimental work. One was the amino acid<sup>1</sup> medium reported previously (2), and the other was the casein hydrolysate medium<sup>2</sup> described by Sprince and Woolley (4). These media were modified as described later.

*Strepogenin Concentrate*—The strepogenin concentrate used throughout the experimental work was prepared as described by Daniel *et al.* (2) from trypsin-digested Labco vitamin-free casein, except that it was not treated with norit. 1 ml. of this concentrate was equivalent to 250  $\gamma$  of casein. The methods employed in the preparation of this strepogenin concentrate have been shown by Sprince and Woolley (11) not to alter the strepogenin activity.

*Analysis for Glutamic Acid and Glutamine*—In view of the findings of Woolley (6) that glutamine and glutamic acid derivatives exhibit strepogenin-like activity, studies were undertaken to assay the strepogenin concentrate qualitatively for glutamine and glutamic acid.

This assay was carried out by the two dimensional paper partition chromatographic method of Consden, Gordon, and Martin (12). The solvents used in the assay were a water-saturated solution of phenol and a mixture of equal parts of crude 2,4,6-trimethylpyridine and 2,4-dimethylpyridine saturated with water. After the two runs with the solvents, the chromatogram was dried in an air stream overnight. A solution of 0.1 per cent ninhydrin in *n*-butanol was then sprayed on the chromatogram and the color was developed in an air oven at 100° for 5 minutes.

Examination of the strepogenin chromatogram, when compared to a reference chromatogram, revealed the presence of both glutamine and glutamic acid in sufficient quantity to give a good ninhydrin color reaction. A further check was made by adding 100  $\gamma$  of L-glutamine<sup>3</sup> to the strepogenin concentrate. This resulted in a more intense color at the characteristic glutamine locus on the chromatogram and showed conclusively the presence of glutamine in the strepogenin concentrate.

In view of these qualitative results, the chemical method of Harris

<sup>1</sup> The authors are indebted to the Herman Frasch Foundation for Chemical Research, care of the United States Trust Company, New York, for some of the amino acids used in this work.

<sup>2</sup> The hydrolyzed casein used as the nitrogen source in the medium was the "vitamin-free" casein hydrolysate of General Biochemicals, Inc., Chagrin Falls, Ohio.

<sup>3</sup> Obtained from B. L. Lemke, New York.

(13) was used to determine the quantity of glutamine present in the strepogenin concentrate. 37  $\gamma$  of glutamine were found in that amount of strepogenin concentrate equivalent to 1 mg. of original vitamin-free casein. The quantity of glutamic acid present in the strepogenin concentrate was determined microbiologically with the use of *Streptococcus faecalis*.<sup>4</sup> Glutamic acid was found to be present to the extent of 45  $\gamma$  per 1 mg. equivalent of strepogenin concentrate.

*Studies with Amino Acid Basal Medium*—The presence of glutamine and glutamic acid in the strepogenin concentrate having been established, studies were undertaken to determine whether these substances would replace strepogenin concentrate as a source of strepogenin activity. A chemically defined basal medium was used to prevent the possible interference of unknown substances which might be present in a casein hydrolysate and to aid in a better understanding of the nature of strepogenin activity.

The results of the first study on the effects of adsorbed and unadsorbed strepogenin concentrate alone and in combination with two sources of liver extracts<sup>5</sup> used in the treatment of pernicious anemia are presented in Table I. When unadsorbed strepogenin concentrate was added alone to the basal medium, a favorable response was obtained. However, the norit-treated strepogenin concentrate showed little activity when added alone to the basal medium at a level of 40 mg. equivalents per tube. Similarly, as shown earlier (2), the addition of the combined antipernicious anemia liver extracts at the level of 0.4 unit per tube promoted very little growth. However, the addition of the norit-treated strepogenin concentrate and liver extracts in combination resulted in maximum growth. L-Glutamic acid<sup>6</sup> also promoted maximum growth in combination with the liver extracts when it was supplied in levels above the 1 mg. per tube already present in the basal medium.

In subsequent studies 0.4 unit per tube of each of the two antipernicious anemia liver extracts was included in the basal medium in order to eliminate any response which might arise from the norit-adsorbable factors present in the unadsorbed strepogenin concentrate. Unless otherwise indicated, the basal medium was also modified by omitting glutamic acid in order that no strepogenin-like response would be obtained from it.

The effects of autoclaving, adsorbing, steaming, and sterilization by filtration on the strepogenin activity of strepogenin concentrate, L-

<sup>4</sup> Daniel, L. J., unpublished data, Cornell University, Ithaca, New York.

<sup>5</sup> Both extracts contained 15 U. S. P. units per ml. One was a product of Eli Lilly and Company, Indianapolis, Indiana, and the other was from Sharp and Dohme, Philadelphia, Pennsylvania.

<sup>6</sup> A product of the Pfanstiehl Chemical Company, Waukegan, Illinois.

glutamine, and L-glutamic acid are presented in Table II. The strepogenin activity of the strepogenin concentrate and L-glutamic acid was found to be approximately the same whether added aseptically or steamed in the medium. When autoclaved for 2 hours at pH 7, however, a loss of strepogenin activity occurred in both materials, but to a greater degree in the strepogenin concentrate. This indicated that the effect of strepogenin was not entirely due to glutamic acid. It is seen from Table II also that

TABLE I

*Strepogenin Activity of L-Glutamic Acid and Strepogenin Concentrate on Amino Acid Medium*

Supplement	Level	Galvanometer reading*
	<i>units</i>	
None		100
Lilly liver extract	0.4	94
Sharp and Dohme liver extract	0.4	80
Combined liver extracts†	0.4 each	82
	<i>mg.</i>	
Strepogenin concentrate (unadsorbed)‡	40	47
"          "          (adsorbed)§	20	95
"          "          "          "	40	94
L-Glutamic acid	8	100
Combined liver extracts		
+ strepogenin concentrate (adsorbed)	20	14
+ "          "          "          "	40	12
+ L-glutamic acid	1	45
+ "          "          "	2	35
+ "          "          "	4	18
+ "          "          "	6	14

\* A galvanometer reading of 100 represents no growth.

† A mixture of equal volumes of Lilly and Sharp and Dohme, 15 U. S. P. units per ml., liver extracts.

‡ Prepared from Labco vitamin-free casein.

§ Adsorbed two times for a duration of  $\frac{1}{2}$  hour each time with an equal weight of norit.

|| L-Glutamic acid neutralized with 1 N NaOH before assay.

very little L-glutamic acid was adsorbed after being treated four times with an equal weight of norit for a half hour's duration each time. Under the same conditions, the strepogenin concentrate was almost entirely inactivated.

On the other hand, when L-glutamine was subjected to these treatments, greater differences were obtained. As shown in Table II, the addition of L-glutamine aseptically to the steamed medium resulted in the highest observed activity, since only 100  $\gamma$  of L-glutamine per tube were required



TABLE II  
*Effects of Various Treatments on Some Strepogenin-Active Substances*

Supplement	Level	Galvanometer reading*			
		Sterile†	Steamed‡	Autoclaved§	Adsorbed
	mg.				
Strepogenin concentrate¶	0	100	100	100	100
" "	0.5		48	100	
" "	1	36	36	93	98
" "	2	24	25	85	97
" "	3		19	63	
" "	4	15	17	47	96
" "	5		16	44	
" "	6	13	15		99
" "	8	12	14		99
" "	10	12	12		92
L-Glutamic acid	0	100	100	100	100
" "	1	85	82	90	90
" "	2	65	67	83	75
" "	4	42	24	38	45
" "	6	18	17	30	27
" "	8	18	14	27	18
" "	10	15	13	25	16
	γ				
L-Glutamine	0	100	100	100	100
" "	25	42			98
" "	50	30			98
" "	75	24			
" "	100	19	47		98
" "	200		40		96
" "	400		34		
" "	500	18			
" "	800		29		
" "	1000	15	27	99	
" "	2000		22	98	
" "	4000	14	21	99	
" "	6000			99	

\* A galvanometer reading of 100 represents no growth.

† Sterilized by passing through a Seitz filter and adding aseptically to the steamed medium.

‡ Steamed in the medium.

§ Autoclaved for 2 hours at 120° and 15 pounds at pH 7, added aseptically to the steamed medium.

|| Strepogenin concentrate and L-glutamic acid adsorbed with an equal weight of norit of  $\frac{1}{2}$  hour duration for four times; then steamed in the medium. L-Glutamine adsorbed with 1 gm. of norit for each 5 mg. for  $\frac{1}{2}$  hour duration for four times; then steamed in the medium.

¶ From Labeo "vitamin-free" casein.

to give maximum growth. This potency was reduced considerably when the *L*-glutamine was steamed in the medium. When *L*-glutamine was autoclaved at pH 7 for 2 hours, or when it was treated four times with 1 gm. of norit per 5 mg. of *L*-glutamine, it was completely inactivated as a source of strepogenin activity.

The reduction in potency of *L*-glutamine which occurs when it is steamed in the amino acid medium as shown here, or when it is autoclaved for 20 minutes in a casein hydrolysate medium as shown by Woolley (6), does not necessarily indicate that the glutamine present in strepogenin concentrates is inactivated in the same manner. McIlwain (14) has shown that *L*-glutamine in some bacteriological culture media containing agar is stable

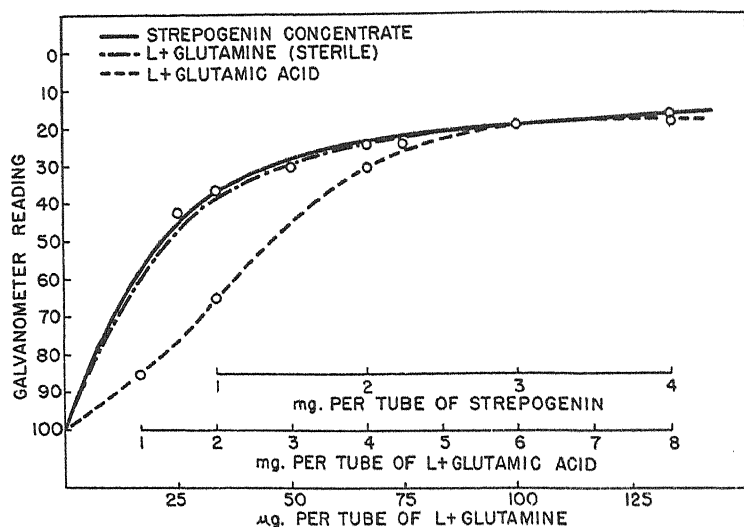


FIG. 1. Strepogenin activity of *L*-glutamine, *L*-glutamic acid, and strepogenin concentrate on an amino acid basal medium.

to autoclaving at 120° and pH 7.2 for 20 minutes. These findings help to explain the partial heat stability of the growth-promoting activity found in strepogenin concentrate.

The data presented in Fig. 1 show the characteristic curves obtained from the addition of these strepogenin-active substances to the basal medium. The addition of strepogenin concentrate resulted in a normal growth curve which reached its maximum plateau in repeated assays with 3 to 4 mg. equivalents of strepogenin. The addition of *L*-glutamine aseptically to the steamed medium promoted growth identical with that of the strepogenin concentrate. The normal growth response was obtained with approximately 100  $\gamma$  per tube of sterile *L*-glutamine. This amount

of glutamine accounted for all the activity of the strepogenin concentrate, since comparison of the responses from strepogenin concentrate and glutamine shows that 33  $\gamma$  per tube of L-glutamine produced the response obtained from 1 mg. equivalent of strepogenin concentrate. This is in good agreement with the 37  $\gamma$  of glutamine per mg. equivalent found by chemical assay to be present in the strepogenin concentrate. Thus the strepogenin concentrate provided 111 to 148  $\gamma$  of glutamine at the level which promoted maximum growth.

L-Glutamic acid promoted growth which reached a maximum value after an initial lag at the lower levels. From 6 to 8 mg. of L-glutamic acid were found to be required to promote maximum growth. The shape of the glutamic acid curve is sigmoidal in character and probably indicates a conversion of glutamic acid to glutamine, the reaction being slower, the smaller the concentration of glutamic acid. The findings here agree with those of Feeney and Strong (15) that glutamine is the key or essential metabolite for *Lactobacillus casei* and can be synthesized by it from glutamic acid.

Since the glutamic acid content of the strepogenin concentrate was found to be 45  $\gamma$  per 1 mg. equivalent, this provided only 0.135 to 0.180 mg. of glutamic acid at the level of strepogenin concentrate which promoted maximum growth. This, therefore, accounted for only a very small part of the observed growth response.

The responses obtained from the addition of sterile L-glutamine to the amino acid basal medium used here do not agree with the findings of Woolley (6) in which sterile L-glutamine added to casein hydrolysate medium produced a definite, but submaximum, response and the curve resulting from the plotted results was not superimposed on the one produced by the Wilson's liver fraction L standard.

Since L-glutamine alone or liver extracts alone produced no growth response on the amino acid basal medium, but resulted in maximum response when added in combination, a significant amount of glutamine cannot be present in the liver extracts.

The responses resulting from L-glutamine and strepogenin concentrate on the amino acid basal medium without L-glutamic acid compared with the responses resulting from their addition to the basal medium containing 1 mg. of L-glutamic acid per tube are presented in Fig. 2. The growth curves for L-glutamine and strepogenin concentrate were still identical. However, on the basal medium containing the added L-glutamic acid, the maximum response was reached in a shorter time, showing the need of the organism for both glutamic acid and glutamine. The level of L-glutamine which produced the same growth as 1 mg. equivalent of strepogenin concentrate was 34  $\gamma$  per tube. This is in agreement with previous results.

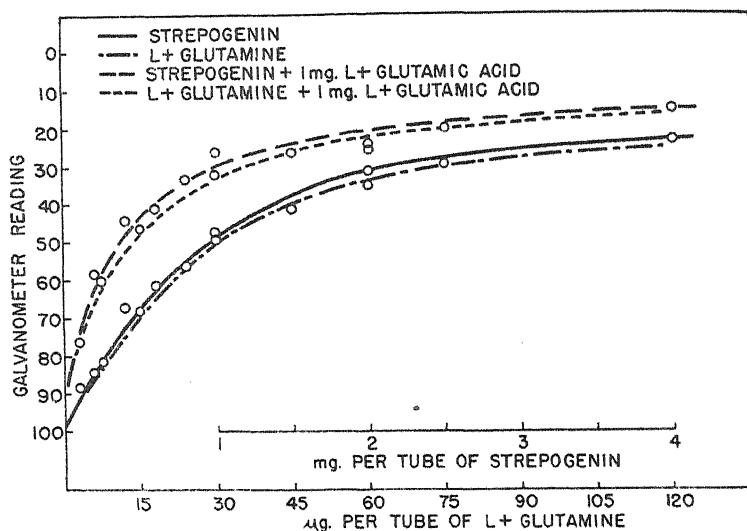


FIG. 2. Comparison of streptogenin activity of L-glutamine and streptogenin concentrate with and without 1 mg. per tube of L-glutamic acid in the medium.

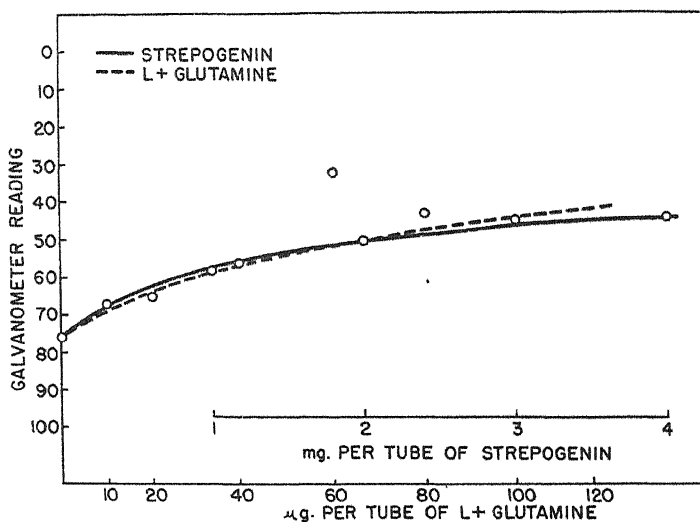


FIG. 3. Streptogenin activity of L-glutamine and streptogenin concentrate on amino acid basal medium containing 6 mg. per tube of L-glutamic acid.

When the basal medium contained 6 mg. of L-glutamic acid per tube, the responses from the addition of streptogenin concentrate and L-glutamine were again similar. Fig. 3 shows the results of this study.

The results presented in Figs. 1 to 3 show that, as the level of L-glutamic acid becomes greater, the response from the strepogenin concentrate or L-glutamine becomes less, and the blank assay tubes become heavier with growth. This probably indicates that the need for glutamine in this organism becomes less as the level of glutamic acid is increased, which again agrees with the suggestion of Feeney and Strong (15) that large quantities of glutamic acid promote a more rapid synthesis of glutamine.

*Studies with Casein Hydrolysate Basal Medium*—In view of the discrepancies in the results obtained with an amino acid medium and those obtained by Sprince and Woolley (11) and Woolley (6) with a casein hydrolysate medium, studies were undertaken with a medium similar to that of Sprince and Woolley (4) in an effort to account for the differences.

The casein hydrolysate basal medium was essentially that described by Landy and Dicken (5). However, it was fortified with 10  $\gamma$  per ml. of  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  (4) in order to avoid a complicating manganese deficiency (16), and was modified to contain the vitamin mixture described by Daniel, Scott, Heuser, and Norris (2) to avoid a deficiency of a known vitamin.

The results of the first study with this medium, presented in Table III, showed that the addition of the combined antipernicious anemia liver extracts promoted the greatest responses, thus indicating potent "strepogenin" activity contrary to the results obtained from adding them alone to the amino acid basal medium. The addition of L-glutamic acid gave no visible growth, whereas supplementation with L-glutamine gave a definite response which reached a plateau at a submaximum growth level. The strepogenin concentrate gave a good growth curve and the response obtained reached a maximum when the concentrate was supplied at the level of 600 to 800  $\gamma$  equivalents per tube.

In view of the fact that L-glutamine promoted a growth response when added aseptically to the casein hydrolysate basal medium, and no growth with the amino acid medium except in combination with the liver extracts, it appeared that the hydrolyzed casein used in the preparation of this medium contained traces of the factors present in the liver extracts. The liver extracts have been shown by Daniel, Peeler, Norris, and Scott (17) to contain at least two unidentified factors other than glutamine, needed by *Lactobacillus casei* for growth. One of these factors has been found to be present in a *p*-cresol extract of liver paste dialysate and the other in a 0.1 N  $\text{H}_2\text{SO}_4$  extract of whey.

A sample of the hydrolyzed casein solution was, therefore, assayed for the presence of these two factors by the method described by Daniel *et al.* (17). The results of the assay indicated that the hydrolyzed casein contains considerable quantities of the factor present in the liver paste dialysate, but that it contains a suboptimum amount of the whey factor.

The submaximum responses arising from the addition of sterile L-glutamine to the casein hydrolysate basal medium appear to be due, at least in part, therefore, to the presence of a limiting amount of the whey factor.

TABLE III  
*Effect of Strepogenin-Active Substances on Casein Hydrolysate Basal Medium*

Supplement	Level	Galvanometer reading*
	<i>units each</i>	
Combined liver extracts†	0.005	82
“ “ “	0.01	76
“ “ “	0.05	25
“ “ “	0.1	15
“ “ “	0.2	11
“ “ “	0.4	9
	<i>mg.</i>	
L-Glutamic acid	1	100
“ “ “	2	100
“ “ “	4	100
“ “ “	6	100
	<i>γ</i>	
L-Glutamine (sterile)	50	90
“ “ “	100	80
“ “ “	200	69
“ “ “	400	47
“ “ “	600	57
“ “ “	800	57
“ “ “	1000	61
Strepogenin concentrate‡	50	85
“ “ “	100	72
“ “ “	200	
“ “ “	400	30
“ “ “	600	16
“ “ “	800	20

\* A galvanometer reading of 100 represents no growth.

† Both extracts contained 15 U. S. P. units per ml. One was a product of Eli Lilly and Company, Indianapolis, Indiana, and the other from Sharp and Dohme, Philadelphia.

‡ Prepared from Labco “vitamin-free” casein.

When no glutamine was present in the casein hydrolysate basal medium, no growth was observed in the blank assay tubes at the usual assay periods of 19 to 23 hours, but maximum growth was attained by the 37th hour of incubation. These results are not surprising, since glutamine is necessary for early and rapid growth of *Lactobacillus casei*, and in its absence poor growth results in the early stages, due to the slow conversion of glutamic

acid to glutamine. It appears probable from these studies, therefore, that the factors present in the liver extracts are synthesized to some extent, and may be concerned with the conversion of glutamic acid to glutamine.

Since glutamic acid did not promote the growth of *Lactobacillus casei*, whereas sterile L-glutamine promoted good growth, it may be that the strepogenin-active peptides of glutamic acid of Woolley (6) can be more easily converted to glutamine than can glutamic acid. This would explain the intermediate response also obtained from glutathione (6).

The activity of L-serylglycyl-L-glutamic acid, as shown by Krehl and Fruton (18), on a synthetic basal medium may be explained, in part, by the effect of the size of inoculum<sup>7</sup> used in the assay. It is possible that sufficient quantities of the liver extract factors were carried over with the inoculum to allow the slight response noted by these workers (18).

In view of the foregoing findings, it is evident that the total activity of strepogenin concentrate prepared from trypsinized casein which Sprince and Woolley (11) reported to be due to "strepogenin" is the effect of more than one factor. Thus, one portion of the strepogenin activity for *Lactobacillus casei* of strepogenin concentrates prepared from casein is distinct from that present in purified antipernicious anemia liver preparations and is proportional to the glutamine content of these concentrates.

#### SUMMARY

Experiments were conducted to show the effects of sterilization by filtration, steaming in the medium, autoclaving, and adsorption on the strepogenin activity of L-glutamine, L-glutamic acid, and strepogenin concentrate.

With a synthetic medium, the growth response to the addition of sterile L-glutamine is identical with that obtained with a strepogenin concentrate. The amount of sterile L-glutamine required to give this growth response is in good agreement with the amount of glutamine found by chemical analysis in the strepogenin concentrate. L-glutamic acid is less active, and its addition resulted in a sigmoidal growth curve, indicating its probable conversion to glutamine.

Evidence was obtained that acid-hydrolyzed casein contains at least two unidentified growth factors which are also present in liver extracts. One is present in considerable quantity, while the other factor is present in suboptimum amounts.

Under the conditions used, *Lactobacillus casei* requires glutamine and glutamic acid in addition to the unidentified factors present in refined liver extracts.

<sup>7</sup> Daniel, L. J., unpublished data, Cornell University, Ithaca, New York.

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# UNIDENTIFIED FACTORS REQUIRED BY *LACTOBACILLUS CASEI*\*

## IV. EVIDENCE FOR TWO GROWTH FACTORS IN PURIFIED LIVER EXTRACTS

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(Received for publication, September 15, 1948)

Scott, Norris, and Heuser (1) and Daniel, Scott, Heuser, and Norris (2) reported that the unknown growth-promoting activity in several natural products for *Lactobacillus casei* was multiple in nature. The latter investigators proposed the existence of two unknown factors as an explanation for the wide variations in response to different antipernicious anemia liver extracts obtained with *L. casei* on an amino acid basal medium containing strepogenin concentrate. Peeler, Daniel, Norris, and Heuser (3) found that one portion of the strepogenin activity of strepogenin concentrates prepared from casein for *L. casei* is distinct from that present in purified antipernicious anemia liver preparations and that it is proportional to the glutamine content of these concentrates.

Studies<sup>1</sup> in this laboratory (4) with chicks fed a soy bean meal diet and with hens fed a purified diet have demonstrated that 95 per cent ethanol-soluble liver paste contains an unidentified factor necessary for chick growth and hatchability. McGinnis and Carver (5) have confirmed this work with chicks. Hill, Scott, Norris, and Heuser (6) reported an unidentified growth factor for chicks fed a soy bean meal diet, in whey, dried distillers' solubles, and dried brewers' yeast. Hill (7) has confirmed these findings by reporting a factor in whey that can be demonstrated with chicks fed a soy bean meal diet, but only when the soy bean meal itself is deficient in the factor. He found that whey produced a suboptimum growth response, whereas fish-meal supported maximum growth. The

\* This work was undertaken in cooperation with the Office of Naval Research, Navy Department, Washington, D. C., and was aided by grants to Cornell University by the Cerophyl Laboratories, Inc., Kansas City, Missouri, the Nutrition Foundation, Inc., New York, and the Western Condensing Company, San Francisco, California. This work was conducted in the Nutrition Laboratories of the Department of Poultry Husbandry.

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<sup>1</sup> Combs, G. F., unpublished data, Cornell University, Ithaca, New York.

hypothesis has been set forth, therefore, that fish-meal contains two factors, one of which is present in whey.

The results of the studies reported in this paper confirm the hypothesis proposed previously (2) and show that *Lactobacillus casei* requires at least two unidentified factors in natural materials in addition to glutamine.

#### EXPERIMENTAL

*Culture*—Descriptions of the method of handling the culture of *Lactobacillus casei*, the preparation of the inoculum tubes, the method of inoculation, the incubation procedure, and the measurement of growth have been presented in detail by Daniel, Scott, Heuser, and Norris (2).

*Medium Used*—An amino acid<sup>2</sup> basal medium was employed in most of the experiments conducted. This medium was identical with that reported previously (2). In view of the activity of glutamine and glutamic acid (3), 4 mg. of L-glutamic acid and 100  $\gamma$  of L-glutamine were added per tube (10 ml.) to the medium.

*Sources of Unidentified Factors*—The materials used as sources for the unknown factors were purified liver extracts, an ethanol-soluble liver paste, and an extract of dried whey.

The antipernicious anemia liver extracts were secured from a local pharmacy with the exception of two samples which were obtained directly from the manufacturer. Seven extracts containing 15 U. S. P. units per ml. and one each of 5 and 10 U. S. P. units per ml. were studied. Dilutions of the extracts were made with water and they were assayed directly.

The liver paste used was the 95 per cent ethanol-soluble product obtained from Wilson and Company, Chicago, Illinois. A dialysate was prepared by dialyzing liver paste in a cellophane bag against distilled water for 3 weeks, with six changes of water. The dialysate was adjusted to pH 5.0, evaporated on a steam bath *in vacuo* to approximately one-tenth of the total volume, and then passed through a column of activated alumina, approximately 1 cm. in diameter and 15 cm. long. The filtrate was extracted with *p*-cresol, and the *p*-cresol removed with diethyl ether. The ether was evaporated and the water solution of the residue was used.<sup>3</sup>

The whey extract was prepared by suspending 5 gm. of dried whey in 200 ml. of 0.1 N H<sub>2</sub>SO<sub>4</sub> and heating it in a boiling water bath for 1 hour. The mixture was cooled, adjusted to pH 4.5 to 5.0 with 1 N NaOH, and filtered through Whatman No. 12 fluted filter paper. It then was adjusted to pH 6.5 to 7.0, steamed 10 minutes, cooled, made to 250 ml., and filtered through Whatman No. 44 filter paper.

<sup>2</sup> The authors are indebted to the Herman Frasch Foundation for Chemical Research, care of the United States Trust Company, New York, for some of the amino acids used in this work.

<sup>3</sup> The authors are indebted to G. F. Combs for this preparation.

## RESULTS AND DISCUSSION

In view of the chick results mentioned earlier in this report,  $\text{H}_2\text{SO}_4$ -extract whey and a water solution of liver paste were studied for their *Lactobacillus casei* activity, alone and in various combinations. The results of this study are presented in Table I.

It is evident from these data that liver paste alone produced appreciable growth of *Lactobacillus casei*, but did not yield a maximum response. Whey extracted alone at the levels used promoted very little growth. However, when whey extract and liver paste were added together, maximum growth occurred. This was not caused by the addition of more of the same factor, since a plateau was obtained with liver paste alone. Furthermore, the turbidity readings obtained on the tubes containing both liver paste and whey extract represent much heavier growth than do the equivalent readings on either of these fractions alone, because the latter readings were made against a basal medium blank in which there was no turbidity, whereas the former readings were made with the corresponding whey extract level as the blank. Therefore, the very heavy turbidity obtained from supplementation with both whey extract and liver paste indicates a definite synergistic effect.

The effect of liver paste dialysate and whey extract on the growth of *Lactobacillus casei* was also studied, and these results are also presented in Table I. The data show more clearly the presence of two factors, inasmuch as the liver paste dialysate itself supported less growth alone than liver paste. This indicates a loss of some whey factor activity by dialysis or adsorption.

The effect of supplementation of the basal medium with liver paste dialysate or whey extract on the response of *Lactobacillus casei* to extracts of various products was studied. Sulfuric acid extracts of these materials were prepared by the procedure used to extract whey. The samples were assayed with unsupplemented basal medium, with whey extract at a level equivalent to 3 mg. of original whey per tube, and with dialysate equivalent to 15 mg. of liver paste per tube. These levels of whey and liver paste dialysate appear to be border line in their content of whey factor and liver paste factor, respectively. However, the use of border line levels was necessary, since increasing either of them produced blanks with considerable growth due to traces of the other factor. The results of this experiment are presented in Table II.

The data indicate that the activity of most of these products was greatly supplemented by the addition of whey extract to the medium, but was considerably less affected, in general, by the addition of liver paste dialysate. The whey factor, therefore, was limiting in most of these products.

In view of these results, it seemed possible that the differences obtained previously (2) in the response of *Lactobacillus casei* to various liver extracts

TABLE I

*Effect of Liver Paste and Whey on Growth of Lactobacillus casei*  
23 hours' incubation.

		Whey†	Galvanometer reading‡
	mg. per tube	mg. per tube	
Liver paste*	0	0	100§
	0	1	93
	0	2	87
	0	3	77
	2.5	0	68
	5	0	57
	10	0	42
	15	0	33
	20	0	44
	2.5	1	67
	5	1	52
	10	1	41
	15	1	27
	20	1	33
	2.5	2	61
	5	2	51
	10	2	35
	15	2	23
	20	2	23
	2.5	3	51
	5	3	38
	10	3	24
	15	3	18
	20	3	16
Liver paste dialy- sate	5	0	96
	10	0	82
	15	0	67
	20	0	59
	5	3	44
	10	3	25
	15	3	23
	20	3	20

\* Water solution of 95 per cent ethanol-soluble Wilson's liver paste.

† H<sub>2</sub>SO<sub>4</sub> extract of dried whey.

‡ A galvanometer reading of 100 represents no growth.

§ The blank is an inoculated tube which contained no growth.

|| The tube containing the proper level of whey alone was set to read 100 on the galvanometer, and then the readings were made on the tubes containing liver paste and that level of whey.

might be attributed to varying degrees of deficiency of the whey factor. This possibility was investigated with the results given in Table III.

TABLE II

*Effect of Whey and Liver Paste Supplements on Response of Lactobacillus casei to Extracts of Natural Products*

21 hours' incubation.

Sample	Level	Supplements to basal medium		
		None	Whey*	Liver paste dialysate†
	<i>mg. per tube</i>			
Brewers' yeast.....	0.5	75‡	44	70
	1	55	26	57
	2	40	21	40
	3	27	18	28
Fish-meal.....	0.5	81	44	78
	1	60	32	55
	2	50	21	43
	3	25	12	23
Liver meal.....	5	64	52	67
	10	48	39	54
	15	35	30	41
	20	31	22	29
Crude casein.....	2.5	65	52	60
	5	48	28	38
	7.5	25	20	25
	10	20	15	19
Whey.....	2	88	51	64
	4	64	40	42
	6	49	24	23
	8	35	20	16
Soy bean meal.....	2.5	85	48	73
	5	58	37	55
	7.5	46	24	42
	10	31	22	31
Liver extract 1§.....	0.025	62	40	63
	0.05	53	27	51
	0.10	27	17	25
“ “ 2.....	0.025	80	50	79
	0.05	68	32	68
	0.10	59	23	59

\* 3 mg. per tube H<sub>2</sub>SO<sub>4</sub>-extracted whey.

† 15 mg. per tube.

‡ Galvanometer readings with corresponding blanks set at 100.

§ Antipernicious anemia liver extracts, both samples of 15 U. S. P. units per ml.

|| Units per tube, instead of mg.

The results of this study show in a striking manner that the assay of the liver extracts on the unsupplemented basal medium measured the whey factor, since practically perfect correlation was found to exist between the

unsupplemented values and those obtained with liver paste dialysate in the medium. Furthermore by the addition of whey to the medium, a reasonable uniformity of response to the different liver extracts on a unit basis resulted. This correlation points to the possibility that the factor in liver extracts, other than the whey factor, to which *Lactobacillus casei* responds may be similar or identical to the factor important in pernicious anemia.

The whey factor activity of soy bean meals was investigated by using a basal medium supplemented with liver paste dialysate. The relative potencies obtained were compared with chick growth on a basal diet com-

TABLE III

*Effect of Whey on Lactobacillus casei Response to Antipernicious Anemia Liver Extracts*

22 hours' incubation.

Liver extract*	Supplements to basal medium		
	None	Whey†	Liver paste dialysate‡
A (15 units per ml.)§.....	100	100	100
B (15 " " " ).....	24	81	24
C (15 " " " ).....	10	76	16
D (15 " " " ).....	30	77	36
E (15 " " " ).....	24	66	20
F (15 " " " ).....	28	67	44
G (15 " " " ).....	35	61	40
H (10 " " " ).....	34	71	32
I (5 " " " ).....	52	103	50

\* Liver extracts of various pharmaceutical companies represented by letters.

† 3 mg. per tube of H<sub>2</sub>SO<sub>4</sub>-extracted whey.

‡ 15 mg. per tube.

§ This liver extract was used as the standard for comparison and was arbitrarily assigned a value of 100. All other values are related to this and may be considered as percentages. All values are on a unit basis.

posed of 33 per cent of the corresponding soy bean meal as reported by Hill (7). The results obtained are presented in Table IV. The correlation between chick activity and whey factor for *Lactobacillus casei* is not perfect, but the trend was definitely present. Indications are strong, therefore, that, when liver paste dialysate is present in the medium, *Lactobacillus casei* responds to an unknown factor needed for the growth of chicks.

Many products were assayed for their whey factor and liver paste factor content by the inclusion of one or the other of the factors in the basal medium. Some of the best sources of the whey factor were found to be

purified liver extracts, dried whey, crude casein, soy bean meal, and dried brewers' yeast, although there were wide variations in the several samples of the yeast analyzed. Water extraction was observed to remove more of the whey factor from casein than ethanol extraction. Liver meal, liver paste, and most fish-meals are relatively poor sources. The factor present in liver paste occurs to a considerable extent in yeast, fish-meal, purified liver extracts, liver paste, blood fibrin, crude casein, and cow manure factor concentrate. Soy bean meal, dried whey, and purified casein are poor sources.

Peeler, Daniel, Norris, and Heuser (3) have pointed out that the casein hydrolysate used in the casein basal medium of Landy and Dicken (8) for *Lactobacillus casei*, when assayed in the amino acid medium for whey factor and liver paste factor, was found to contain considerable liver paste

TABLE IV  
Correlation between "Whey Factor" Activity for *Lactobacillus casei* and Chick Growth Obtained on Soy Bean Meals

Sample of soy bean meal	<i>L. casei</i> *	Chick growth 6 wks.
	mg. units whey per gm.†	gm.
A	710	425
B	790	454
C	740	477
D	920	509
E	1050	521
F	940	525
G	990	532

\* Medium contained liver paste dialysate at a level equivalent to 15 mg. of liver paste per tube.

† Based on dried whey as 1000 mg. units per gm.

factor activity and little whey factor activity. Scott, Norris, and Heuser (1) used this casein basal medium to assay many natural products for factor S or "strepogenin" activity. Since the most limiting of the unidentified factors in the casein hydrolysate was the whey factor, a study was undertaken to determine the relative potency of several natural products when assayed on both media for whey factor activity. The results of this study are presented in Fig. 1.

Considerable correlation exists between the results obtained on the same sample assayed on the two media. Thus, the use of the casein hydrolysate basal medium results in an assay primarily for the whey factor. There are some discrepancies obtained, especially in the case of soy bean meal and whey. Both of these products are relatively low in the factor present in liver paste, so that it is possible that the casein medium is

border line in this factor, and when an insufficient amount is present in the sample a lowered response is obtained.

Under the experimental conditions used by Scott, Norris, and Heuser (1), in which the casein hydrolysate was norit-treated, it is probable that the assay was measuring two factors, but since in most products the whey factor is the limiting one, the over-all effect was probably that of a whey factor response.

Factor S, the chick growth factor described by Schumacher, Heuser, and Norris (9), and Hill, Scott, Norris, and Heuser (6), which Scott, Norris, and Heuser (10) correlated with the *Lactobacillus casei* activity of products

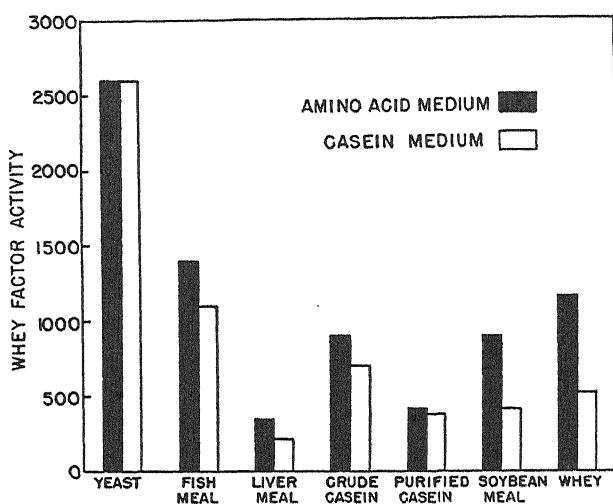


FIG. 1. Whey factor activity of natural products obtained by growth of *Lactobacillus casei* on two media. Whey activity has been calculated by comparing potency with that of whey extract which has been assigned a value of 1000 mg. units per gm.

assayed on a casein medium, may be the same or similar to the whey factor activity for *Lactobacillus casei* herein described.

Excellent correlation was obtained between the chick growth factor in liver paste and the *Lactobacillus casei* activity as more purified concentrates were prepared. However, upon application of the counter-current distribution technique of Craig (11) to the liver paste dialysate, fractions were obtained in which the chick and bacterial growth factors were separated. Under the present experimental conditions, it is doubtful whether *Lactobacillus casei* is measuring the "animal protein factor" of the chick.

In view of our present knowledge of the unidentified factors required by *Lactobacillus casei*, it is apparent that for optimum growth the organism



requires at least three substances, glutamine, whey factor, and liver paste factor. On the basis of this information, the identity of strepogenin in relation to these factors is rather confused.

Sprince and Woolley (12) determined strepogenin activity by the response of *Lactobacillus casei* to tryptic digests of materials on an acid-hydrolyzed casein medium. Peeler *et al.* (3) found this medium to be border line in liver paste factor, but deficient in whey factor and glutamine, so that a response might be due to one or both of these factors. Woolley (13) also reported glutamine to possess the highest strepogenin activity for *L. casei*, yet to be completely inactive in promoting a growth response in mice (14). The strepogenin concentrate made from casein was highly active for the mouse and *L. casei*. Since crude casein or trypsin-digested casein contains a considerable amount of the unidentified factors needed by *L. casei*, it is probable that one of these substances was stimulating mouse growth.

Therefore, strepogenin activity may have been produced by one or more of these factors, depending upon whether the mouse or microorganism was used for assay purposes.

No attempt has been made to relate these factors to any of the other unidentified factors required by other bacteria and animals.

#### SUMMARY

It has been shown that *Lactobacillus casei* requires at least two factors in addition to glutamine for rapid and complete growth. One of these factors exists to a large extent free of the other factor in liver paste dialysate. The other, or whey factor, is present in whey extract relatively free of the liver paste factor. The response of *Lactobacillus casei* to most natural materials thus far investigated is limited by the amount of whey factor present rather than the amount of liver paste factor, with the exception of soy bean meal and whey.

Antipernicious anemia liver extracts assayed on an unsupplemented amino acid medium gave responses with *Lactobacillus casei* similar to those obtained with liver paste in the medium. When whey was added to the medium, a rather uniform response was obtained from all extracts on a unit basis. It appears, therefore, that these purified liver extracts are relatively more deficient in whey factor than in the liver paste factor, and that the variable responses of *Lactobacillus casei* to the unsupplemented extracts obtained in this work and previously (2) were caused by differences in the amounts of whey factor present in the various liver extracts.

It seems probable that assays made on a casein medium are assays for the whey factor, since acid-hydrolyzed casein is deficient in this factor. Thus the chick activity produced from soy bean oil meals and other sources of factor S may be caused by the presence of whey factor.

Present experimental evidence suggests that the factor present in liver paste which is active in promoting growth of *Lactobacillus casei* is not the same as the "animal protein factor" for chicks.

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# A STUDY ON THE SYNTHESIS OF CYSTINE BY SOME LACTIC ACID BACTERIA\*

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(Received for publication, October 1, 1948)

The strains of *Lactobacillus arabinosus* 17-5, ATCC 8014, *Lactobacillus casei*, ATCC 7469, *Lactobacillus casei* var. *delbrueckii* LD5, ATCC 9595, and *Leuconostoc mesenteroides* ATCC 68100, which have been kept in the stock culture collection of the Department of Bacteriology at the University of Illinois, were found to require cystine but not methionine for maximal growth. This result was considered interesting in view of the essential nature of methionine in the diet of animals and the more frequent need of preformed methionine in bacteria. There is some evidence in the literature which would seem to indicate that in bacteria the two sulfur-containing amino acids are metabolically related through intermediary steps analogous to those postulated by Binkley and du Vigneaud (1) for animals and demonstrated in *Neurospora* mutants by Horowitz (2). Of interest in this connection are the papers by Lampen, Roepke, and Jones (3) and Simmonds (4), both dealing with mutants of *Escherichia coli*. Riesen, Schweigert, and Elvehjem (5) and Stokes, Gunness, Dwyer, and Caswell (6), working with lactic acid bacteria, found that methionine was not replaceable by homocystine, cystathionine, cystine, cysteine, choline, or glutathione among other sulfur-containing substances.

The experiments reported in this paper were designed to analyze the cystine requirements of the strains of lactic acid bacteria listed above. Stock cultures of these organisms were grown in stab culture on a medium consisting of 1 per cent tryptone, 0.5 per cent glucose, 0.5 per cent yeast extract, and 1 per cent agar. They were transferred every 2 months and stored in the refrigerator. No difficulty was encountered as far as contamination or variations in the cultures were concerned. All organisms grew well at 37°, with the exception of *Leuconostoc mesenteroides*, which had to be incubated at 28°.

In order to obtain inocula the organisms were subcultured in tryptone broth (1 per cent tryptone, 0.5 per cent glucose, 0.5 per cent yeast extract), incubated 20 to 24 hours, washed three times, and suspended in distilled water. The density of the suspensions was adjusted to give a turbidity barely discernible by the naked eye and a reading of about 95 to 97 in a

\* Summary of a thesis presented in partial fulfillment of the requirements for the degree of doctor of philosophy by the senior author.

Lumetron colorimeter when the instrument was set to read 100 with distilled water. 0.01 ml. of such a suspension served as the inoculum for each test-tube.

All the glassware employed in the experiments was cleaned in dichromate cleaning solution and rinsed thoroughly in tap and distilled water. The reagents were crystalline products of Eastman Kodak Company, Merck and Company, Inc., and Biochemicals, Inc., with the exception of leucine, isoleucine, methionine, tyrosine, alanine, phenylalanine, and serine, which had been prepared by the Department of Chemistry of the University of Illinois.

The basal medium and the technique used were those of Dunn, Shankman, Camien, and Block (7) with slight modifications. The basal medium had the composition indicated in Table I. In the case of *Leuconostoc mesenteroides* the sodium chloride concentration had to be raised to 50 mg. per cent in order to allow maximal growth. 1 ml. amounts of a basal medium were pipetted into suitable test-tubes by means of volumetric pipettes. Then, allowance being made for the volume of the test substances which were to be added after sterilization, water in amounts varying from 0.5 to 0.1 ml. was pipetted into each tube, so that the final volume in each tube prior to inoculation would be 1.5 ml. The tubes were stoppered with cotton plugs and were autoclaved at 15 pounds for 15 to 20 minutes.

DL-Methionine, L-cystine, L-cysteine hydrochloride, sodium thioglycolate, glutathione, and DL-homocystine were tested for their growth-promoting effect in concentrations which were equivalent to 167  $\gamma$  of cystine per 1.5 ml. of final medium on the basis of their sulfur content. They were sterilized separately by autoclaving or filtration through Seitz filters and were added aseptically to the sterile basal medium with the aid of a 0.1 ml. pipette. In addition to the compounds listed above methyl mercaptan, thiourea, sodium taurocholate, sulfanilamide, sodium sulfide, sodium sulfite, and sodium hydrogen sulfate were tested in corresponding concentrations, but were found to be either without effect or inhibitory to the growth of the organisms.

After inoculation the cultures were incubated for 72 hours, after which time they were heated in flowing steam for 10 to 15 minutes to arrest growth. They were titrated immediately in order to determine the amount of growth which had taken place. With brom-thymol blue as an indicator, standard sodium hydroxide (0.017 to 0.019 N) was added from a micro burette directly to the culture in the culture tube until the color matched that of a phosphate buffer solution of pH 7.0. All the tests were made in duplicate; the results were recalculated to read as ml. of a 0.02 N solution of sodium hydroxide and could then be averaged.

TABLE I  
Composition of Basal Medium

	mg. per cent		
DL-Alanine	16.7	Calcium pantothenate	200
L-Aspartic acid	16.7	Pyridoxine	160
L-Arginine hydrochloride	16.7	Biotin	0.5
L-Glutamic acid	16.7	Folic acid	0.5
Glycine	16.7	p-Aminobenzoic acid	10.0
L-Histidine hydrochloride	16.7		
Hydroxy-L-proline	16.7		mg. per cent
DL-Isoleucine	16.7	Inositol	2.5
L-Leucine	16.7		gm. per cent
DL-Lysine monohydrochloride	16.7	Glucose	2.0
DL-Phenylalanine	16.7	Sodium acetate	1.2
L-Proline	16.7		
DL-Threonine	16.7		mg. per cent
L-Tryptophan	16.7	Ammonium sulfate	740.0
L-Tyrosine	16.7	Potassium dihydrogen phosphate	50.0
DL-Valine	16.7	Dipotassium hydrogen phosphate	50.0
Adenine sulfate	1.3	Sodium chloride	35.0
Guanine	1.3	MgSO <sub>4</sub> ·7H <sub>2</sub> O	20.0
Uracil	1.3	MnCl <sub>2</sub> ·4H <sub>2</sub> O	1.0
Xanthine	1.3	FeSO <sub>4</sub> ·7H <sub>2</sub> O	1.0
	γ per cent		
Thiamine hydrochloride	100		
Nicotinic acid	200		
Riboflavin	200		

pH 6.8 to 6.9.

TABLE II  
Effect of Various Sulfur-Containing Compounds on Growth of Test Organisms in Amino Acid Medium with or without Serine

Final volume per culture tube = 1.5 ml. Results expressed in ml. of 0.02 N sodium hydroxide.

	<i>L. mesenteroides</i>		<i>L. arabinosus</i>		<i>L. casei</i>		<i>L. delbrueckii</i>	
	With serine	Without serine	With serine	Without serine	With serine	Without serine	With serine	Without serine
Basal medium.....	2.5	2.4	4.4	3.7	1.4	0.5	1.3	0.6
+ cystine.....	5.5	5.0	8.0	8.6	5.1	0.7	5.3	0.7
+ cysteine.....	5.2	4.9	9.0	8.4	5.7	0.6	6.2	0.7
+ glutathione....	5.4	4.7	9.6	8.5	5.5	0.7	6.6	0.6
+ thioglycolate....	2.2	2.4	4.5	3.0	3.1	0.7	3.5	0.7
+ homocystine....	4.7	4.1	8.3	5.2	3.6	0.8	4.1	0.8

The experiments were then repeated with a basal medium which contained 167  $\gamma$  per 1.5 ml. of serine in addition to the other amino acids, the experimental technique and calculations being the same as those indicated above.

The results are summarized in Table II.

#### DISCUSSION

An analysis of the data reveals that cystine, cysteine, and glutathione bring about the maximal growth of *Lactobacillus arabinosus* and *Leuconostoc mesenteroides* regardless of the presence or absence of serine. Homocystine, on the other hand, exhibits a comparable degree of stimulation only if serine is furnished in the basal medium. The partial effect produced by homocystine in the absence of serine is probably due to endogenous serine synthesis. This assumption is supported by the fact that *Lactobacillus arabinosus*, which is mildly responsive to preformed serine, is also only weakly reactive to homocystine alone, while *Leuconostoc mesenteroides*, which has no serine requirement, reacts almost maximally to homocystine alone. There is no response to the addition of thioglycolate. These results would suggest that these two bacterial strains have a specific cystine demand and are able to synthesize cystine from homocystine in the presence of serine and, therefore, presumably via cystathionine. Unfortunately, the latter compound was not available for study.

In the case of *Lactobacillus casei* and *Lactobacillus delbrueckii* serine was found to be absolutely essential and thioglycolate and homocystine brought about a partial stimulation only as compared to the maximal growth which took place in the presence of cystine, cysteine, and glutathione. These results, therefore, do not throw much light on the probable pathway of cystine synthesis in these organisms and do not exclude the possibility of a non-specific demand for sulfhydryl groups.

Methionine alone or in combination with ethanolamine did not stimulate the growth of any of the four test organisms and, consequently, also, homocystine with or without choline did not produce any effect beyond that due to its cystine activity. Since methionine has been found to be present in the protoplasm of lactic acid bacteria (8) and, since the strains studied here do not require this amino acid, they must be able to synthesize it. On the other hand, methionine is unable to serve as cystine precursor. The conclusions to be drawn from this are either that these organisms are unable to demethylate methionine or that they demethylate it to something other than homocysteine or a similar suitable cystine precursor. Simmonds (4), whose paper appeared after the completion of the experiments reported here, suggests the possibility that the conversion of methio-

nine to cystine involves intermediary steps which are different from those concerned with the transformation of cystine into methionine. This remains an alternative hypothesis. There are some indications in the literature to the effect that some bacteria may be unable to utilize methionine, choline, or betaine as sources of methyl groups, for instance the pneumococcus work of Badger (9) and the *Escherichia coli* studies of Green and Sevag (10). On the other hand, Doudoroff (11) and Starr (12) have obtained evidence for the methylation of homocystine in a mutant strain of *Photobacterium phosphoreum* and certain *Xanthomonas* strains.

#### SUMMARY

Strains of *Lactobacillus arabinosus* and *Leuconostoc mesenteroides* which require cystine but not methionine for maximal growth have been found to be able to utilize homocystine as a cystine substitute if serine is supplied in the basal medium. The results have been interpreted as indicating the inability of these bacterial strains to transform methionine into homocystine, while demonstrating their ability to couple homocystine to serine to form cystine. Similar conclusions could not be reached in the case of strains of *L. casei* and *L. delbrueckii* because of the serine demand of these organisms.

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# DETERMINATION AND PROPERTIES OF AN UNIDENTIFIED GROWTH FACTOR REQUIRED BY LACTOBACILLUS BULGARICUS\*

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(Received for publication, October 7, 1948)

Past developments in biochemistry serve to emphasize the similarity in the nutritional requirements of animals and microorganisms and thus lend added interest to a study of unidentified growth factors for bacteria. Discovery of a strain of *Lactobacillus bulgaricus* which appeared to require a hitherto undescribed growth essential present in yeast extract has been described briefly (1). The same organism required oleic acid (2) in addition to the customary ingredients of the medium, and was greatly stimulated by lactose or other  $\beta$ -galactosides (3, 4). The assay method for the unidentified growth factor, procedures for its partial purification, and certain properties of the concentrates obtained are described below.

## EXPERIMENTAL

*Stock Cultures and Inoculum*—The test organism, a strain of *Lactobacillus bulgaricus*,<sup>1</sup> was isolated from milk. Stock cultures are carried by weekly transfer in litmus milk. 24 hours before an assay is planned a small wire loopful of the milk culture is transferred to 10 ml. of the single strength basal medium supplemented with 10 mg. of yeast extract. After incubation for 24 hours at 37°, the cells are centrifuged, washed twice with sterile saline, and finally resuspended in 10 ml. of sterile saline. 1 drop of this suspension is used to inoculate each assay tube of 10 ml.

*Basal Medium*—The composition of the basal medium is shown in Table I. In addition to the usual ingredients it contains oleic acid, which

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by grants from Merck and Company, Inc., and from the Division of Research Grants and Fellowships, National Institutes of Health, United States Public Health Service. Portions of this work were presented before the Fourth International Congress for Microbiology, Copenhagen, July 20–26, 1947 (1).

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<sup>1</sup> We are indebted to Professor W. B. Sarles for cultures of this organism and information concerning its classification.

is required by the test organism (2), Tween 40,<sup>2</sup> which serves to detoxify the oleic acid (2), and lactose, which is required for rapid growth of the

TABLE I  
*Composition of Basal Medium*

Component	Amount per liter of double strength medium	Component	Amount per liter of double strength medium
	gm.		gm.
Acid-hydrolyzed casein*.....	10	Asparagine.....	0.2
Enzymatic digest of casein†...	20	Cystine.....	0.2
Acetic acid‡.....	13.3	Cysteine.....	0.2
Glucose.....	20	Tween 40.....	2.0
Lactose.....	20	Oleic acid.....	0.02
KH <sub>2</sub> PO <sub>4</sub> .....	2		mg.
K <sub>2</sub> HPO <sub>4</sub> .....	2	Pyridoxal hydrochloride.....	0.4
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.4	Thiamine chloride.....	0.4
NaCl.....	0.02	Calcium pantothenate.....	0.8
FeSO <sub>4</sub> ·7H <sub>2</sub> O.....	0.02	Riboflavin.....	0.8
MnSO <sub>4</sub> ·4H <sub>2</sub> O.....	0.02	Niacin.....	0.8
Adenine.....	0.02	p-Aminobenzoic acid.....	0.4
Guanine.....	0.02	Pteroylglutamic ".....	0.032
Uracil.....	0.02	Biotin.....	0.004

\* 100 gm. of Labco casein plus 500 ml. of 20 per cent HCl refluxed for 24 hours. The hydrolysate is evaporated *in vacuo* to a thick syrup and 500 ml. of distilled water are added and removed *in vacuo*. This is repeated three to five times. The solution is finally adjusted to pH 3.5 and diluted to 1 liter. This is stirred with 20 gm. of Darco G-60 at room temperature for 30 minutes. The pH is adjusted to 6.8 and the charcoal treatment repeated. 1 ml. of the final solution is equivalent to 100 mg. of casein.

† 120 gm. of Labco casein are suspended in 2 liters of 0.8 per cent NaHCO<sub>3</sub> and adjusted to pH 8.0 with KOH. 2 gm. of pancreatin (Merck) dissolved in 20 ml. of water are added to the mixture, which is then incubated for 48 hours at 37° under a layer of benzene. The digest is filtered through Filter-Cel, 80 gm. of glacial acetic acid are added, and the solution is diluted to 2.4 liters. 1 ml. of this solution is equivalent to 50 mg. of casein and it actually should contain by test 40 to 50 mg. of solids per ml. The digest is stored at 4° under benzene until use.

‡ This amount of acetic acid is supplied by the enzymatic digest of casein, when used in the amounts indicated and prepared as described above.

test organism (3, 4). An enzymatic casein digest was added to the medium to avoid possible interference from peptides such as streptogenin (5), al-

<sup>2</sup> A polyoxyethylene derivative of sorbitan monopalmitate supplied by the Atlas Powder Company. Tween 80 (the corresponding monooleate) can be used in place of Tween 40 plus oleic acid if desired (2). Tween 40 is preferred, since it does not become rancid on storage.

though it is not known whether or not such peptides play a rôle in the nutrition of this organism. To avoid formation of emulsions of the basal medium with the preserving solvent during storage, oleic acid and Tween 40 are added just before use from a solution containing 2 gm. of Tween 40 and 20 mg. of oleic acid in 20 ml. of water. 1 ml. of this solution per 50 ml. of double strength medium will give the prescribed amounts. The pH of the stock solution of the basal medium (without Tween 40 or oleic acid) is adjusted to 6.5 with potassium hydroxide, and stored at 4° under a thin layer of benzene. The medium can be kept successfully in this fashion for at least 2 months.

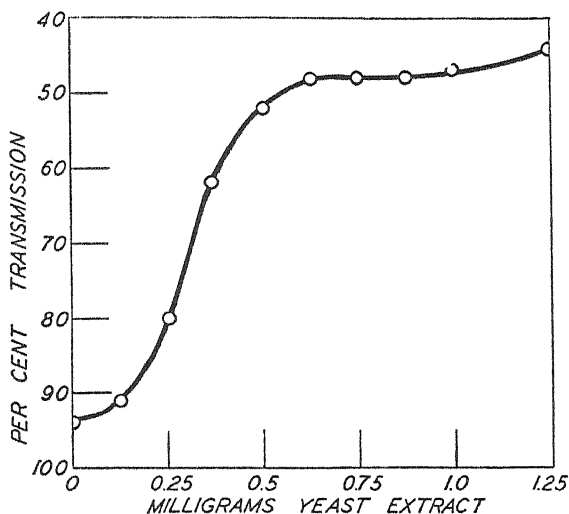


FIG. 1. Response of *Lactobacillus bulgaricus* to additions of yeast extract in the deficient medium.

*Assay Procedure for Lactobacillus bulgaricus Factor (LBF)*—Yeast extract is a good source of the unidentified factor and has been used as an arbitrary standard in this investigation. Graded amounts of a solution of yeast extract are added to a series of tubes containing 5 ml. of the double strength basal medium and the volume in each tube is adjusted to 10 ml. with water. The tubes are covered with aluminum caps and autoclaved at 15 pounds pressure per sq. in. for 6 minutes. After cooling, each tube is inoculated with 1 drop of the inoculum suspension and incubated at 37° for 19 to 24 hours. Growth is estimated with the Evelyn colorimeter and the 660 m $\mu$  filter. Fig. 1 shows the growth response after 19 hours. Tubes containing no yeast extract or suboptimal amounts will frequently grow to maximum in 48 hours. If the inoculum is transferred twice at 24

hour intervals in the inoculum medium before use, this effect is eliminated and no growth without yeast extract occurs even after 96 hours of incubation.

A unit of LBF has been defined for convenience as that amount of the growth factor contained in 1 mg. of standard yeast extract (Basamin Busch, manufactured by Anheuser-Busch, Inc.). This amount gives approximately maximum growth when contained in 10 ml. of medium. Samples for assay are diluted to contain an estimated 0.25 to 0.5 unit per ml. and assayed at five levels. Their potency is calculated in the customary fashion, and expressed in terms of units per mg. or ml. of sample. Ethanol or butanol extracts can be assayed directly if not more than 0.1 ml. of the alcohol is added per assay tube.

TABLE II  
*Distribution of Growth Factor*

Material tested	Potency	Material tested	Potency
	<i>units per mg. solids</i>		<i>units per mg. solids</i>
Yeast extract (Anheuser-Busch)	1.0	Whey solids	0.2
Yeast extract (Difco)	0.85	Curbay B. G.	0.35
Dried yeast (Anheuser-Busch)	0.5	Florida blackstrap molasses	0.00
Butanol extract of yeast extract	10.0	Corn steep liquor	0.36
Butanol extract of malt sprouts	3.0	Whole liver	0.00
Butanol extract of lyophilized liver	1.6	Liver fraction I.	0.25
Clarase	0.7	“ powder 1-20	0.2
Tomato juice (centrifuged)	0.3	“ extract (Sharp and Dohme, 15 U. S. P. units per ml.)	3.0
Cabbage (blended and centrifuged)	0.3		

*Distribution of Growth Factor*—Results of assay of a variety of products for LBF are shown in Table II. Water extracts were assayed in all cases, although the inactivity of whole liver extracts as contrasted with the activity of various liver fractions suggests that the factor occurs in some materials in a “bound” form. It is obvious that the factor is widely distributed in natural materials and hence is probably of general metabolic significance. Of the crude materials tested, yeast extract is the richest source of the factor, and was therefore used as a starting material in attempts at concentration.

*Concentration of LBF from Yeast Extract*—A variety of procedures has been tried in attempts to purify the growth factor. One of the most successful sequences of steps so far developed is outlined below.

1. Extraction with butanol. 9.5 liters of *n*-butanol plus 0.5 liter of water were heated to 97–98°. 1 kilo of yeast extract (Basamin Busch) was slowly added to the vigorously stirred solution. The mixture was stirred under a reflux for 40 minutes, cooled to room temperature, and the insoluble residue separated by centrifugation and filtration. The residue was resuspended in 1 liter of dry butanol, stirred briefly at 98°, and filtered. (a) Residue, 313,000 units, discarded; (b) combined butanol extract, 63.1 gm., 632,000 units, concentration 10-fold.

2. Water extraction of butanol extract. The combined butanol extract (Step 1, *b* above, 8.42 liters) was stirred vigorously at 70° with 3.4 liters of water for 15 minutes and the two phases separated. This water extraction was repeated twice with 1.7 liters of water each time and at a temperature of 80°. (a) Combined water phases, 48.2 gm., 191,000 units, discarded; (b) butanol phase, 16.6 gm., 485,000 units, concentration 29-fold.

3. Evaporation under a vacuum. The butanol phase (Step 2, *b*, 7.24 liters) was evaporated to dryness under reduced pressure. The temperature in the water bath should not exceed 60°. The residue was dissolved in 300 ml. of hot butanol. After standing below 0° for 48 hours, a precipitate was filtered in the cold. (a) Precipitate, 3.1 gm., 22,800 units, discarded; (b) butanol solution, 13.23 gm., 365,000 units, concentration 28-fold.

4. Adsorption on charcoal. The butanol solution (Step 3, *b*) was further evaporated to a volume of 80 ml., then filtered through a column of activated charcoal (Darco G-60) 20 cm. in height and 5.70 cm. in diameter. The column was treated in turn with 500 ml. of propanol, 500 ml. of a propanol-*n*-butanol mixture (1:1), and finally 4000 ml. of *n*-butanol containing 1 per cent of pyridine. (a) First 1230 ml. of filtrate, 6.25 gm., 41,690 units, concentration 26-fold; (b) remaining 3190 ml. of eluate, 1.89 gm., 159,000 units, concentration 84-fold.

5. Adsorption on magnesium silicate. The butanol solution (Step 4, *b*) was evaporated to dryness and redissolved in 50 ml. of warm butanol. This was adsorbed on a column of magnesium silicate-Filter-Cel (1:2) 16 cm. in height and 1.55 cm. in diameter. The column was then treated in turn with 500 ml. of dry *n*-butanol, 100 ml. of *n*-butanol containing 1 per cent water, 100 ml. of *n*-butanol containing 5 per cent water, and 200 ml. of *n*-butanol containing 10 per cent water. (a) First 520 ml. of eluate, 1.13 gm., 23,900 units, concentration 21-fold; (b) remaining 320 ml. of eluate; 0.375 gm., 58,400 units, concentration 156-fold.

The over-all yield of activity obtained in the purest fraction, Step 5, *b*, was thus only 5.8 per cent of that in the starting material. The best concentrates so far available were obtained by minor variations of the above procedures, and were concentrated about 300-fold over the starting material. Such fractions, which are obviously far from pure, are brown-

colored gums and permit maximum growth of the test organism when added to the basal medium in amounts of 4  $\gamma$  per 10 ml. At this stage of purity they are thus somewhat more active on a weight basis in promoting growth than are the most active amino acids (e.g., a variety of lactic acid bacteria requires 25 to 30  $\gamma$  of tryptophan per 10 ml. to promote maximum growth (6)), but are still considerably less active than the least potent of the vitamins (e.g., *Lactobacillus casei* requires 0.3 to 0.5  $\gamma$  of riboflavin per 10 ml. of medium to promote maximum growth (7)).

*Stability and Miscellaneous Properties of LBF*—At room temperature the growth factor appears reasonably resistant to alterations in pH between 2 and 10. At elevated temperatures it is much less stable. For example, a solution of the purified growth factor (200 times concentrated) was autoclaved at 15 pounds pressure per sq. in. for 2 hours. The per cent of the initial activity which remained was as follows: pH 1.0, 0.0; pH 2.0, 10; pH 4.0, 66; pH 7.0, 66; pH 8.8, 48; pH 11, 26. It is not destroyed by digestion with proteolytic, saccharolytic, or phosphorolytic enzymes (e.g., trypsin, papain, clarase). In preliminary tests, the active factor has not been destroyed by treatment with nitrous acid. Extraction tests from buffers with butanol and ether indicate that it is neither strongly acidic nor strongly basic.

*Specificity of Requirement for New Growth Factor*—A large number of physiologically important compounds have been tested for possible growth-promoting activity for *Lactobacillus bulgaricus* under the conditions of assay for the unknown factor. These included lecithin, 2-methylnaphthoquinone, Synkamin (a water-soluble derivative of vitamin K), ergosterol, cholesterol, DL-alanine, DL-pantoinine,<sup>3</sup> ribonucleic acid, desoxyribonucleic acid, uridine, adenosine, guanosine, cytidine, inosine, adenine desoxyriboside, thymidine, hypoxanthine, pyridoxamine phosphate, coenzymes I and II, coenzyme A,<sup>3</sup> asparagine, glutamine, glutathione,  $\alpha$ -tocopherol phosphate, choline, inositol, putrescine, spermine, spermidine, ascorbic acid, and ergostanyl acetate.<sup>3</sup> None of these showed any trace of activity except ergostanyl acetate.

As shown in Table III, ergostanyl acetate produces a peculiar type of delayed response. At 19 hours this compound fails to stimulate significant growth, while a concentrate of the growth factor permits good growth in 16 to 19 hours. After 44 hours high levels of ergostanyl acetate caused only an incomplete growth response. A preparation of "crystalline guinea pig factor" (the antistiffness factor) isolated from natural sources<sup>3</sup> showed the same type of activity. Interpretation of these results is complicated by

<sup>3</sup> We are indebted to Dr. T. H. Jukes for ergostanyl acetate, to Dr. A. L. Caldwell for the isolated crystalline guinea pig factor, to Dr. E. L. R. Stokstad for a concentrate of Kidder's Factor II, to Dr. W. Shive for a sample of DL-pantoinine ( $\alpha$ -amino- $\beta$ , $\beta$ -dimethyl- $\gamma$ -hydroxybutyric acid), and to Dr. Fritz Lipmann for coenzyme A.

the insolubility of these compounds, and, possibly for this reason, maximum growth could not be obtained with them. Since, in any case, these materials (known to cure a syndrome produced in guinea pigs (8, 9)) are less than one three-hundredth as active on a weight basis as the best concentrates obtained from yeast, they cannot be the active agent in the concentrates. A functional relationship to the growth factor may, however, be indicated. A concentrate of Factor II,<sup>3</sup> required for growth of *Tetrahymena geleii* (10), was also inactive.

*Possible Identity with Other Unidentified Growth Factors*—It does not appear profitable to speculate on the possible identity of this substance with other uncharacterized growth factors reported in the literature. The high activity of yeast extract and the comparatively low activity of liver concentrates used in treatment of pernicious anemia (Table II) indicate that the growth factor cannot be identical with vitamin B<sub>12</sub>. Furthermore,

TABLE III  
Comparison of Growth Response of *L. bulgaricus* to Concentrate of LBF and Ergostanyl Acetate

Weight of solids in concentrate (260-fold)	Growth response*		Weight of ergostanyl acetate	Growth response*	
	19 hrs.	44 hrs.		19 hrs.	44 hrs.
$\gamma$			$\gamma$		
0.0	96	94	0.0	96	94
0.6	86	83	100	96	94
1.2	82	79	200	96	83
2.4	65	57	400	96	75
4.8	58	45	800	92	60

\* Per cent of incident light transmitted, distilled water = 100.

concentrates of the factor are inactive in replacing the requirement of *Lactobacillus lactis* for liver extracts. Similarly, concentrates of LBF are inactive in replacing growth factors described for *Streptococcus faecalis* (11) and for *Leuconostoc citrovorum* (12). As mentioned earlier, adequate sources of streptogenin and the known vitamins are present in the basal medium. Thus the factor appears not to have been described previously.

*Other Organisms Requiring LBF*—Three of six additional strains of *Lactobacillus bulgaricus* tested appear to require the unidentified growth factor. A strain of *Lactobacillus helveticus* also responded to a concentrate of the growth factor. Preliminary evidence indicates that it may be widely required by fastidious organisms of this group.

#### SUMMARY

Conditions are described which permit detection and assay of a new essential growth factor required by certain strains of *Lactobacillus bul-*

*garicus* and *Lactobacillus helveticus*. The factor is widely distributed in natural materials, yeast extracts being a particularly rich source. By extraction with butanol, followed by fractional extraction with water and adsorption and elution from charcoal and magnesium silicate, concentrates were obtained which were from 150 to 300 times as active as yeast extract in promoting growth of the test organism. 4  $\gamma$  of such concentrates per 10 ml. of medium permitted maximum growth.

The new growth factor is readily destroyed by alkali and acid. It could not be replaced by a number of physiologically active compounds tested, and appeared to differ from other unidentified growth factors described previously. Ergostanyl acetate and a crystalline fraction from natural materials which possess "antistiffness" activity in guinea pigs evoked a delayed and incomplete growth response of the test organism at high levels. They were less than one three-hundredth as active in this respect as the best concentrates of the growth factor, and their relationship to it, if any, is not known.

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## CARBAMATES IN THE CHEMOTHERAPY OF LEUCEMIA

### IV. THE DISTRIBUTION OF RADIOACTIVITY IN TISSUES OF MICE FOLLOWING INJECTION OF CARBONYL-LABELED URETHANE

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(Received for publication, October 18, 1948)

Haddow (1) in a recent review on the chemotherapy of cancer stated that, "Although a great deal of work has been recorded on the suppressive action of urethane, phenyl urethane, and related carbamates on other growth processes, as in bacteria, protozoa, sea urchin eggs, plant tissues, and animal tissues growing *in vitro*, little of this is of direct assistance in suggesting the mechanism by which urethane produces its effects in leukemia." With the availability of radioactive urethane we have undertaken two types of tracer investigations in hopes of shedding some light on this important subject. The first studies, a portion of which are reported herein, were concerned with the possibility that urethane is exerting its anti-leucemic action on a particular organ, tissue, or system (limited in a gross anatomical sense) and that such action might be detected by specific accumulation or fixation of the compound or a radioactive atom thereof. The second phase of this investigation, which will be reported elsewhere, was directed toward tracing the radioactive portion of the urethane molecule into various cell constituents (nuclei, chromosome fractions, cytoplasmic fractions, etc.) which could be isolated by physical and chemical procedures prior to activity assays.

Although it was considered unlikely from the onset of this program that urethane would exhibit tissue or organ specificity, the lack of any information on this point and the possibility that hematopoietic tissue or tumor tissue of neoplastic animals might preferentially localize the whole or a portion of the urethane molecule suggested the necessity of this work.

No attempts have been made in this particular study to distinguish between fixed and free urethane or urethane decomposition products in tissues. Hence, these results are a measure of the presence of the carbonyl carbon from urethane in the tissues at the time the animals were sacrificed. A portion of the tissue activity is almost surely the result of fixation of the  $C^{14}O_2$  which is produced from catabolism of carbonyl-labeled urethane.

In these initial experiments considerable effort has been made to account for the total activity injected. The expired  $CO_2$ , the urine, and the feces have been collected and representative samples of the most interesting tissues taken for subsequent oxidation and activity assay. The rate and

extent of *in vivo* degradation of the urethane molecule have been ascertained by careful measurement of the rate of appearance of radioactive carbon in the respired carbon dioxide.

TABLE I  
*Rate of Appearance of  $C^{14}$  in Respiratory Carbon Dioxide of Normal Mice  
(Cumulative Per Cent of Total)*

Period	Experiment 1	Experiment 2
<i>min.</i>		
0-30	6.2	2.0
30-60	9.7	4.7
<i>hrs.</i>		
1- 2	18.8	10.1
2- 3	26.8	15.9
3- 4	33.3	21.3
4- 5	40.1	27.3
5- 6	45.9	33.1
6-24		99.7

TABLE II  
*Recovery of  $C^{14}$  in Respiratory Carbon Dioxide of Mice Injected with Carbonyl-Labeled Urethane*

Experiment No.	Condition of mice	Per cent of total activity recovered in 24 hrs.
2	Normal	99.7
3	"	92.8
5	"	93.1
11	"	90.0
7	Spontaneous lymphoid leucemia	79.5
9	" " "	77.1
14	" mammary carcinoma	55.8
12	" " "	106.8
16	Methylcholanthrene-induced mammary carcinoma	44.2
13	Induced myeloid chloroleucemia, 1394	96.0

#### EXPERIMENTAL

*Radioactive Urethane*—The carbonyl-labeled urethane,  $\text{NH}_2\text{C}^{14}\text{OOC}_2\text{H}_5$ , used in this study was prepared by a procedure worked out by Bryan.<sup>1</sup> This material, having a specific activity of 51 millicuries per mole, was made up in distilled water so that 0.2 ml. of solution contained a dosage of about 1400 mg. per kilo (78 per cent of the  $\text{LD}_{50}$ ) and a total activity of 2.0

<sup>1</sup> Bryan, C. E., unpublished data.

microcuries. The dosage in mg. per kilo of urethane has varied somewhat with the weight of animals used, but the total radioactivity injected has been maintained at a constant level. All injections have been made by the intraperitoneal route.

TABLE III  
*Radioactivity of Urine*

In Experiments 1, 2, 3, 5, 6, and 11, normal CFW mice were used; in Experiments 7, 8, 9, and 15, Akm mice with advanced spontaneous lymphoid leucemia; in Experiments 12 and 14, CFW mice with spontaneous mammary carcinomas; in Experiment 13, an Akm mouse with induced myeloid chloroleucemia 1394 (11 days after inoculation with leucemic spleen mince).

Experiment No.	Sample voided	Specific activity	Fraction of total C <sup>14</sup>
	<i>hrs.</i>	<i>microcuries per mole C</i>	<i>per cent</i>
1*	After 8	85.8	3.70
2	7½	71.8	3.70
	11½	24.9	5.65
	24	1.49	0.01
			(9.36)†
3	12	44.3	6.12
	21	11.0	0.96
			(7.08)†
5	18	10.5	4.91
6	10 ca.	57.0	9.35
	26 "	1.79	0.60
			(9.95)†
7	13 "	61.8	10.46
	13-24	32.3	7.74
			(18.20)†
8*	5½	88.4	4.13
9	?	24.5	9.08
11	0-24	54.0	8.50
12	0-24	18.0	4.95
13	0-24	42.0	4.20
14	0-24	58.9	8.26
15	0-24	31.3	7.46

\* Died.

† Total for experiment.

*Collection of Carbon Dioxide Samples*—Immediately after injection of the active compound, the mice were placed in a metabolism chamber and the expired CO<sub>2</sub> collected over varying periods, according to a procedure already reported (2).

*Treatment of Tissues*—After a given period the injected mice were removed from the metabolism chamber and anesthetized with ethyl ether,

TABLE IV  
*Specific Activity of Tissue from Mice Injected with Carbonyl-Labeled Urethane*

The results are expressed in microcuries per mole.

In Experiments 2, 3, and 11, normal CFW mice, 24 hours after injection, were used; in Experiments 5 and 6, normal CFW mice, 48 hours after injection; in Experiments 7 and 9, Akm mice with advanced spontaneous lymphoid leucemia, 24 hours after injection; in Experiments 8 and 10, Akm mice with advanced spontaneous lymphoid leucemia (these two mice died 5 to 5½ and 2½ hours after injection, respectively); in Experiments 12 and 14, CFW mice with spontaneous mammary carcinomas, 24 hours after injection; in Experiment 13, an Akm mouse with induced chloroleucemia 1394, 24 hours after injection; in Experiment 15, an Akm mouse with spontaneous lymphoid leucemia, 48 hours after injection; in Experiment 16, a CFW mouse with methylcholanthrene-induced mammary carcinoma.

Tissue	Experiment No.														
	2	3	5	6	7	8	9	10	11	12	13	14	15	16	
Blood.....	0.25	0.53	0.20	0.25	1.00		1.46		0.20	0.29	0.18	7.98	0.03	4.18	
“ serum.....										0.11		1.45			
“ cells.....															
Spleen.....	0.23	0.16	0.15	0.23	0.88	7.81	1.01	8.70	0.10	0.16	0.17	2.04	0.09	3.27	
Adrenals.....	0.08	0.85	0.17	0.06	0.24	1.21	0.14	0.49	0.04	0.10	0.15	0.18	0.07	0.43	
Kidneys.....	0.19	0.16	0.11	0.18	0.87	5.87	1.13	4.18	0.13	0.14	0.14	2.72	0.08	3.52	
Liver.....	0.30	0.54	0.19	0.19	1.01	6.44	0.83	8.89	0.31	0.26	0.19	1.68	0.17	2.70	
Testes.....	0.15	0.13	0.11	0.10	0.78		1.03	6.86	0.09		0.08				
Thymus.....	0.16	0.14	0.14	0.05	0.71	7.73	1.24	10.99	0.04	0.14	0.11	0.30	0.07	0.06	
Heart.....	0.12		0.09	0.07	0.57	6.08	0.90	7.44	0.10					2.68	
Lungs.....	0.14	0.14	0.09	0.07	0.54	6.23	0.92	8.98	0.13			1.23	0.08	3.02	
Lymph nodes.....	0.10	0.11	0.08	0.04	0.82	7.42	0.87	5.69	0.12	0.13	0.10	1.12	0.11	3.02	
Brain.....	0.18	0.07	0.06	0.05	0.74	5.81	0.84	7.08	0.06	0.05	0.06	1.86	0.03	2.50	
Muscle.....	0.07	0.11	0.04	0.05	1.08	2.44	0.96	4.43	0.07	0.06	0.03	2.18	0.02	2.40	
Stomach and small intestine.....	0.43	0.28	0.10	0.14	0.93	8.86	1.34	8.07							
Large intestine.....	0.30	0.30	0.15	0.07	1.08	6.84	1.33	3.46	0.44				0.06	2.61	
Jejunum.....									0.40	0.22	0.26	2.10	0.13	3.42	
Skin and hair.....	0.16	0.05	0.06	0.03	0.36	4.48	0.38	1.85	0.10			1.27	0.02	1.24	

Bone (with marrow).....	0.25	0.11	0.13	25.9	2.72	3.36	0.25	0.34	0.19	1.06	0.08	1.19
Gallbladder and bile.....			0.04					0.01		3.62		0.53
Pancreas.....					2.98							
Uterus.....					7.57			0.25		2.28	0.06	0.19
Ovaries.....										0.53	0.04	
Tumor.....					7.51*			0.22		4.35	0.13*	4.30
“ fluid.....										5.06		
Metastasis.....										2.50		
Lung metastasis.....										1.89		0.64

\* Lymphoid tumors in mesenteric tissue.

The skin was then laid back over the thoracic cavity and a 0.5 ml. sample of blood aspirated from the right ventricle. The blood was in certain instances separated into cells and serum by centrifugation. The tissues of interest were immediately excised, weighed, and stored in small vials at  $-10^{\circ}$  until they could be oxidized and the  $\text{CO}_2$  precipitated therefrom as  $\text{BaCO}_3$ . The procedure for oxidation and precipitation has recently been described (2).

*Radioactivity Assays*—All measurements of radioactivity reported herein were carried out by the procedure of Miller (3), in which  $\text{CO}_2$  is liberated

TABLE V

*Total  $\text{C}^{14}$  Remaining in Animal after Periods Indicated*

In Experiments 2, 3, 5, 6, and 11, normal CFW mice were used; in Experiments 7, 8, 9, 10, and 15, Akm mice with advanced spontaneous lymphoid leucemia; in Experiments 12 and 14, CFW mice with spontaneous mammary carcinomas; in Experiment 13, an Akm mouse with induced chloroleucemia 1394; in Experiment 16, a CFW mouse with methylcholanthrene-induced mammary carcinoma.

Experiment No.	Period of experiment	Fraction of total $\text{C}^{14}$ in all tissues
	<i>hrs.</i>	<i>per cent</i>
2	24	1.4
3	24	1.9
5	48	0.8
6	48	0.7
7	24	10.9
8	5½	72.0 <i>ca.</i>
9	24	10.2
10	2½	82.9
11	24	0.6
12	24	1.6
13	24	0.9
14	24	32.7
15	48	0.4
16	24	46.0

from the radioactive barium carbonate and is introduced directly into the Geiger-Müller tube along with a small quantity of  $\text{CS}_2$  to improve the counting properties. The details of the procedure have been reported previously (2).

*Animals Used*—Normal CFW strain white mice of about 25 gm. weight have been used as controls in these experiments. Akm strain mice of about 30 gm. with spontaneous lymphoid leucemia, CFW strain mice weighing 30 and 40 gm. with spontaneous mammary carcinoma and methylcholanthrene-induced mammary carcinoma, and one Akm mouse (weight 25 gm.) with induced myeloid chloroleucemia 1394 have been the examples of neoplasia studied to date.

### Results

Data with reference to the rate of appearance of radioactive carbon in the respiratory carbon dioxide of mice injected with carbonyl-labeled urethane are summarized in Table I. These results are presented in terms of the cumulative percentage of the total injected activity recovered after the indicated periods. The variation in recovery of  $C^{14}$  by the respiratory route in normal and neoplastic mice as of 24 hours is indicated in Table II.

The activities of urine samples collected during the various experiments are listed in Table III.

TABLE VI  
*Total Recovery*

In Experiments 2, 3, 5, 6, and 11, normal CFW mice were used; in Experiments 7, 9, 10, and 15, Akm mice with advanced spontaneous lymphoid leucemia; in Experiments 12 and 14, CFW mice with spontaneous mammary carcinomas; in Experiment 13, an Akm mouse with induced chloroleucemia 1394; in Experiment 16, a CFW mouse with methylcholanthrene-induced mammary carcinoma.

Experiment No.	Period of experiment	Fraction of total $C^{14}$				
		Per cent expired	Per cent in all tissues	Per cent in urine	Per cent in feces	Per cent of total
	<i>hrs.</i>					
2	24	99.7	1.4	9.4		110.5
3	24	92.8	1.9	7.1		101.8
5	48	93.1	0.8	4.9		98.8
6	48	98.7	0.7	10.0		109.4
7	24	80.1	10.9	18.2	$4.5 \times 10^{-1}$	109.7
9	24	77.4	10.2	9.1		96.7
10	2½	7.0	82.9			89.9
11	24	89.6	0.6	8.5	$6.0 \times 10^{-4}$	98.7
12	24	107.0	1.6	5.0	$5.8 \times 10^{-6}$	113.6
13	24	96.0	0.9	4.2	$9.1 \times 10^{-4}$	101.1
14	24	56.2	32.7	8.3	$2.2 \times 10^{-2}$	97.2
15	48	94.5	0.4	7.5	$5.7 \times 10^{-6}$	102.4
16	24	44.2	46.0			90.2

The specific activities (microcuries per mole of carbon) of the many tissues studied in normal and neoplastic mice are presented in Table IV. While the over-all range of error encountered with regards to total  $C^{14}$  recovery was of the order of  $\pm 15$  per cent, this comparison of tissue activities represents an accuracy of a considerably higher degree. Depending on the  $C^{14}$  content and the selected counting time, the per cent statistical error involved in tissue activity assays varied from  $\pm 1$  to  $\pm 10$  per cent with a majority of the values represented by the lower extreme of the range. The total  $C^{14}$  retention (in all tissues) calculated from total weights of excised organs and tissues of animals studied is shown in Table V.

One of the principal objectives of these exploratory experiments has been to account for the total active carbon injected and thus establish the general mode of excretion of the carbonyl carbon of ethyl carbamate. Table VI indicates the percentage recovery of the total injected activity in the urine, feces, respiratory carbon dioxide, and in all tissues at the indicated intervals and the over-all total recovery in individual experiments.

#### DISCUSSION

The rate of appearance of radioactive carbon in the respiratory carbon dioxide of mice injected with carbonyl-labeled urethane as shown in Table I is an indication of the rate of catabolism of this molecule in the body. These data are in agreement with information gained in this laboratory on the disappearance of free urethane (as determined by chemical analysis) from the whole blood of rabbits and mice.<sup>2</sup> In studies of the clearance rate of  $C^{14}O_2$  from blood of mice, we have observed that 66 per cent of the total activity of a dose of  $NaHC^{14}O_3$  was expired in 10 minutes after injection, and about 93 per cent could be accounted for in the respiratory carbon dioxide in 1 hour. Therefore, it appears that once the labeled portion of the molecule has been degraded to  $C^{14}O_2$ , there is little lag before expiration of the labeled atom.

It is interesting to note the failure of certain neoplastic animals to dispose of the radioactive carbonyl carbon from urethane as rapidly as did normal mice (Table II). Two mice with advanced spontaneous lymphoid leukemia, one mouse with spontaneous mammary carcinoma, and one with methylcholanthrene-induced mammary carcinoma expired considerably less of the labeled carbon than did normal mice over a 24 hour period. These neoplastic mice were all found to have a correspondingly higher tissue retention of the carbon atom in question.

As can be seen in Table III, a small portion of the total activity injected appeared in the urine. The specific activity of the urine samples was at a peak at about 6 to 9 hours and decreased at a constant rate for the remainder of a 24 hour experiment. Chemical analyses indicated that some of this radioactivity of the urine is due to free urethane.

Hawkins and Murphy (4) and Dury and Robin (5) have observed hypertrophied adrenals in rats following administration of urethane. Dustin (6) has reported that urethane causes unusual cellular degeneration in the Lieberkuehn glands of the intestine of the mouse. Nettleship and Henshaw (7) and Larsen (8) have shown that urethane will induce lung tumors in a certain strain of mice. The effects of urethane and other carbamates on the hematopoietic system have also been reported (4, 9). These reports and others too numerous to mention with regards to temporary experi-

<sup>2</sup> Hutchison, O. S., unpublished data.



mental and clinical remissions in leucemia following administration of urethane led us to the selection of leucemic animals for tracer study and suggested tissues of primary interest.

As is shown in Table IV, the active carbon atom from carbonyl-labeled urethane which remains in the body at 24 and 48 hours after injection is fairly well distributed through most of the tissues with little indication of consistent localization. In Experiment 3, with a normal CFW strain mouse, an unusually high value was obtained for the adrenals and in the first leucemic animal, Experiment 7, a very high specific activity was obtained in the sample of bone, which also included bone marrow. Inability to repeat these early suggestions of specificity forces us, at least for the present, to consider them as anomalies.

Further consideration of the data summarized in Table IV indicates that the blood was in most cases comparatively high in radioactivity, and that the serum activity was higher than that of the cells. Measurements of the whole blood bicarbonate in one experiment (normal mouse 24 hours after injection with labeled urethane) showed that this fraction contained but about 5 to 10 per cent of the radioactivity of the blood.<sup>2</sup>

The consistently high activity of the intestines as compared to other tissues is of special interest in the light of the work of Dustin referred to above; however, we also have observed high fixation of  $C^{14}$  from  $NaHC^{14}O_3$  in this tissue.

The tumor tissues have also tended to be among the loci of higher radioactivity, but it cannot be suggested from these data that there is any urethane specificity for malignant cells.

Perhaps the most interesting observation in the present study is the much greater retention of radioactivity in the tissues of two mice with spontaneous lymphoid leucemia and two mice with mammary carcinoma when compared with control mice 24 hours after injection. Since no attempts were made in these experiments to remove free urethane before tissue assays, it is impossible at present to state whether these considerable differences between certain normal and neoplastic animals were due to a greater fixation of urethane or a metabolite therefrom by the neoplastic mice or whether these mice simply failed to catabolize the compound as rapidly as did normal mice. This point is now being studied.

It seems probable that much of the radioactive carbon found in the shorter term experiments (Table IV) was present as free urethane. A portion of the tissue activity in all experiments was undoubtedly due to entrance of  $C^{14}O_2$  from the degraded urethane molecule into the metabolic pathways.

An observation possibly related to the high tissue retention of the radioactive carbonyl carbon from urethane by certain animals with leucemia and mammary tumors was that these particular animals exhaled less

CO<sub>2</sub> per gm. of body weight during a 24 hour period than did normal animals injected with equivalent amounts of urethane.

#### SUMMARY

1. On intraperitoneal injection, urethane begins to break down almost immediately, with the labeled carbonyl carbon being excreted largely as expired carbon dioxide. In normal mice usually greater than 90 per cent of this atom from urethane can be accounted for in the respiratory carbon dioxide within 24 hours. About 5 to 10 per cent of the total activity was found in the urine.

2. A small fraction of the active carbon from carbonyl-labeled urethane is present in all tissues at 24 and 48 hours after injection. In experiments to date, blood serum has been found to contain more active carbon than blood cells. Whole blood, liver, intestines, and tumor tissues have been in a number of cases somewhat high in activity as compared to other tissues.

3. Two mice with advanced spontaneous lymphoid leukemia and two with mammary carcinoma have been found to retain much more radioactive carbon from active urethane in all tissues than have normal mice 24 hours after injection. It has not yet been determined whether this difference is due to failure of the neoplastic animals to catabolize the urethane as rapidly as do normal mice or whether there is greater fixation of urethane (or a urethane metabolite) by the neoplastic animals.

This work was supported by grants from Mr. Ben May and associates of Mobile, Alabama, and the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council. The authors also wish to acknowledge the technical assistance of the following in carrying out various aspects of this program: for the dissections, Mrs. Anne S. White; for tissue oxidations, Mrs. Olivia S. Hutchison and Miss Dorris Shumpert; for radioactivity assays, Mr. Walter Robinson and Mr. Charles H. Schneider.

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# PARTIAL SYNTHESIS OF COMPOUNDS RELATED TO ADRENAL CORTICAL HORMONES

## XI. REACTIONS OF RING C KETOLS IN THE PREPARATION OF AN 11-KETO BILE ACID\*

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(Received for publication, August 27, 1948)

The present investigation was undertaken to provide a method for the preparation of an 11-keto bile acid having no other substituent in Ring C, since this product could serve as a suitable starting material for the partial synthesis of adrenal cortical steroids. In addition, the results provide direct proof for the structure which had been deduced for 3( $\alpha$ ),12( $\beta$ )-dihydroxy-11-ketocholanic acid (1). The essential facts bearing upon the solution of these problems have been briefly reported (2) and the reactions are summarized in Fig. 1. 3( $\alpha$ ),12( $\beta$ )-Dihydroxy-11-ketocholanic acid was converted to the 3-monosuccinate and this product was esterified to the dimethyl ester (I). The free hydroxyl group at C-12 was replaced with halogen on treatment with  $PBr_3$  and the bromo keto ester (II) was reduced to the 11-keto compound (III) with either zinc and acetic acid or with chromous chloride. Several other methods for the removal of the C-12 hydroxyl group were investigated and are described in the experimental section; the procedure which has been outlined provided the best results. The resultant product was identified as the 11-keto acid and the aim of the investigation had thus been accomplished. Since the reactions used were unlikely to cause any extensive alteration of the ketol, they provide independent proof for the presence of an 11-keto group in the initial product.

Since 3( $\alpha$ ),12( $\beta$ )-dihydroxy-11-ketocholanic acid is the principal product resulting from the isomerization of any Ring C ketol under the influence of hot aqueous base, this reaction has been studied in detail. It could be presumed that isomerization of any of the cyclic ketols represented in Fig. 2 would yield all four possible diastereoisomers. The

\* The work reported here was supported in part by a grant from Memorial Hospital, for which we wish to express our thanks.

This paper represents a portion of a thesis submitted by Evelyn Borgstrom to the Division of Biological Sciences of the University of Chicago in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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published work, however, would indicate that only 3( $\alpha$ ),12( $\beta$ )-dihydroxy-11-ketocholanic acid is obtained in considerable amount. The explanation for this apparent oversight is not difficult to find, since an easily crystallized reaction product is so readily obtained. This fact diverted the attention of investigators from the more important observation that the product is a mixture and from this mixture a homogeneous substance is obtained only after sensible loss has been sustained in recrystallization.

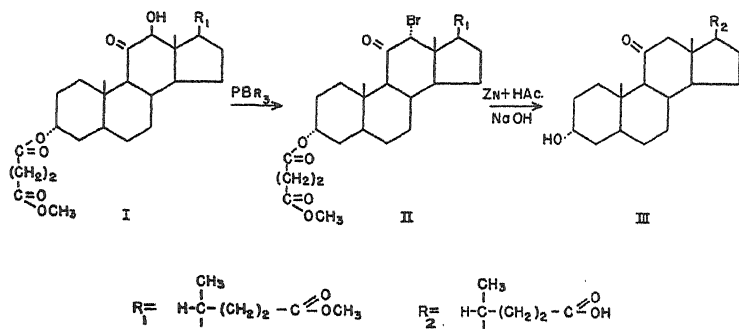


FIG. 1

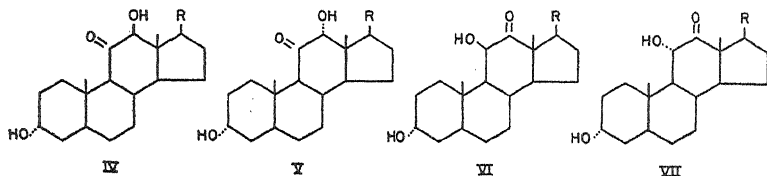


FIG. 2

When the ketol was prepared by hydrolysis of the 11-bromo-12-keto derivative, it was assumed that this loss could be ascribed to inclusion of unchanged keto acid and dehydrobromination or dehydration products formed in the course of the reaction. When, however, the ketols (VI) and (VII) were isomerized to the supposedly stable isomer (IV) in yields of less than 70 per cent, this explanation was no longer tenable and a more critical study of the alkaline isomerization was necessary. This has been done by the methods described in the experimental section, and it has been possible to separate the four compounds shown in Fig. 2 and to recover

about 95 per cent of the material subjected to rearrangement. While the existence of a true equilibrium has not been demonstrated, since the reaction was investigated only for a single compound, it is probable that, if a true equilibrium exists, it is not greatly different from that which has been established under our experimental conditions. The chemical properties of the rearrangement products shown in Fig. 2 have been investigated and have been shown to agree with the structures assigned to these compounds.

#### EXPERIMENTAL<sup>1</sup>

*Isomerization of Ring C Ketol with Alkali*—25.35 gm. of 3( $\alpha$ ),12( $\beta$ )-dihydroxy-11-ketocholanic acid (m.p. 206–208°) were dissolved in 1200 ml. of 5 per cent aqueous sodium hydroxide. The solution was flushed with a stream of nitrogen for  $\frac{1}{2}$  hour and then was heated under a reflux condenser in an atmosphere of nitrogen for 2 hours. The alkaline solution was added slowly to a large volume of dilute aqueous sulfuric acid at  $-5^\circ$  and the partially crystalline precipitate was removed. This was washed by suspension in ice water until the washings were neutral and dried at room temperature. The acid was crystallized from 125 ml. of ethyl acetate and 11.9 gm. were obtained in the first crop. Previous experiments had shown that the product obtained from this solvent was a mixture, but a preliminary separation by crystallization was advantageous because of the great difference in solubility between 3( $\alpha$ ),12( $\beta$ )-dihydroxy-11-ketocholanic acid and the other isomers. The mother liquors were concentrated to dryness and the residue weighed 13.12 gm. The total material recovered was 25.02 gm. and the percentage composition of the mixture was calculated from this weight. The first crop was recrystallized from 95 per cent ethanol, and two crops, totaling 10.40 gm. (41.6 per cent), of 3( $\alpha$ ),12( $\beta$ )-dihydroxy-11-ketocholanic acid with a melting point of 202–204° were obtained.

The mother liquors from these crystallizations were combined with the principal residue of 13.12 gm. and esterified with methanol and sulfuric acid at room temperature; the small acid fraction was converted to the methyl ester with diazomethane. The ester was acetylated by the procedure of Whitman and Schwenk (3) at a temperature below 10°. The product was crystallized from methanol and yielded 6.82 gm. of methyl 3( $\alpha$ ),11( $\alpha$ )-diacetoxy-12-ketocholamate, m.p. 151–152° (21.9 per cent). The non-crystalline residue was hydrolyzed under conditions which had previously been shown to cause no rearrangement of the ketol. 0.5 mm of the methyl ester diacetate in 100 ml. of 0.1 N NaOH in 50 per cent

<sup>1</sup> All melting points are corrected.

ethanol was allowed to stand for 3 hours at 25°, and the reaction mixture was acidified at -5°, saturated with sodium chloride, and extracted with several portions of ether. After washing and removal of the solvent, the product was crystallized from both ethyl acetate and from 95 per cent ethanol as before, yielding 3.55 gm. (14.2 per cent) of 3( $\alpha$ ),12( $\beta$ )-dihydroxy-11-ketocholanic acid with a melting point higher than 202°. Re-esterification and acetylation of the mother liquors and crystallization from methanol yielded an additional 2.0 gm. (6.45 per cent) of methyl 3( $\alpha$ ),11( $\alpha$ )-diacetoxy-12-ketocholanoate. The total amount of crystalline material accounted for 84.15 per cent of the product obtained after isomerization.

*Methyl 3( $\alpha$ ),12( $\alpha$ )-Diacetoxy-11-ketocholanoate*—The residues (4.07 gm.) were chromatographed on 50 times the weight of alumina. Benzene-petroleum ether, 2:1, eluted 1.65 gm. which were dissolved in a small volume of methanol, inoculated with a crystal of methyl 3( $\alpha$ ),12( $\beta$ )-diacetoxy-11-ketocholanoate, and stored at -5° overnight. Two crops of crystals totaling 1.62 gm. and melting at 86-88° were obtained (5.2 per cent). Elution of the chromatogram with benzene gave 1.06 gm., from which 383 mg. of methyl 3( $\alpha$ ),11( $\alpha$ )-diacetoxy-12-ketocholanoate were isolated. Elution with benzene and 2 per cent ether through benzene and 20 per cent ether yielded 380 mg. of an oil which crystallized from petroleum ether. Recrystallization gave 309 mg. of rosettes of slender prisms, m.p. 131-132°. A mixture with methyl 3( $\alpha$ ),11( $\alpha$ )-diacetoxy-12-ketocholanoate, m.p. 151-152°, melted at 114-116° after softening at 103°. Three recrystallizations from petroleum ether yielded methyl 3( $\alpha$ ),12( $\alpha$ )-diacetoxy-11-ketocholanoate melting at 133-134°;  $[\alpha]_D^{22} = +118.1^\circ$  (chloroform).

C<sub>29</sub>H<sub>44</sub>O<sub>7</sub>. Calculated, C 69.02, H 8.83; found, C 69.25, H 8.70

The non-crystalline mother liquors were rechromatographed and additional small amounts of the three isomers described were obtained. When the first eluates containing methyl 3( $\alpha$ ),12( $\beta$ )-diacetoxy-11-ketocholanoate were crystallized, a second crop melted at 90-93°, which is higher than that of the purest sample of methyl 3( $\alpha$ ),12( $\beta$ )-diacetoxy-11-ketocholanoate. Since methyl 3( $\alpha$ ),11( $\beta$ )-diacetoxy-12-ketocholanoate is eluted from alumina in the same solvent mixtures as methyl 3( $\alpha$ ),12( $\beta$ )-diacetoxy-11-ketocholanoate, the mother liquors from these crystals were inoculated with a very small amount of methyl 3( $\alpha$ ),11( $\beta$ )-diacetoxy-12-ketocholanoate. After standing several days, long needles were obtained which appeared to be impure methyl 3( $\alpha$ ),11( $\beta$ )-diacetoxy-12-ketocholanoate. Hydrolysis of the amorphous residues by the procedure previously described, followed by solution of the acid in 1 N sodium hydroxide and

storage at  $-5^{\circ}$  for several days, yielded a small amount of insoluble sodium salt. Since the formation of an insoluble sodium salt is characteristic of  $3(\alpha),11(\beta)$ -dihydroxy-12-ketocholanic acid, this may be considered additional evidence for the presence of this fourth isomer, at least in trace amounts. Table I summarizes the percentages of the four constituents of the isomerization obtained by isolation of the constituents.

*Methyl  $3(\alpha),12(\beta)$ -Diacetoxy-11-ketocholananate*—In order to characterize the esters obtained from the isomerization reaction and to obtain sufficient product for the hydrolytic experiments, the methyl ester diacetate of highly purified  $3(\alpha),12(\beta)$ -dihydroxy-11-ketocholanic acid was prepared by the procedures which have been described. The product was crystallized from cold methanol and melted at  $83-85^{\circ}$  after preliminary softening at  $79^{\circ}$ , and was unchanged after several recrystallizations from the same solvent;  $[\alpha]_D^{22} = +55.4^{\circ}$  (chloroform). The melting point varied by a

TABLE I

*Products Obtained after Isomerization of  $3(\alpha),12(\beta)$ -Dihydroxy-11-ketocholanic Acid with Hot NaOH*

Acid	Per cent isolated
$3(\alpha),12(\beta)$ -Dihydroxy-11-ketocholanic.....	63.1
$3(\alpha),11(\alpha)$ -Dihydroxy-12-ketocholanic.....	30.4
$3(\alpha),12(\alpha)$ -Dihydroxy-11-ketocholanic.....	1.1
$3(\alpha),11(\beta)$ -Dihydroxy-12-ketocholanic.....	Trace
Total.....	94.6

few degrees, depending upon the rate of heating. The lower melting point recorded in the literature (4) may have been due to minor contamination with methyl  $3(\alpha),11(\alpha)$ -diacetoxy-12-ketocholananate.

*Methyl  $3(\alpha),11(\beta)$ -Diacetoxy-12-ketocholananate*—This derivative was prepared from the acid in the same way as methyl  $3(\alpha),12(\beta)$ -diacetoxy-11-ketocholananate. The hydroxy keto acid was obtained by acidification of the insoluble sodium salt described by Gallagher and Long (5) and the methyl ester diacetate was chromatographed in order to free it from the methyl ester acetate of  $3(\alpha)$ -hydroxy-12-ketocholanic acid, which is always coprecipitated with the insoluble sodium salt. A clean separation was obtained by chromatography over 50 times the weight of alumina and the product obtained proved to be the methyl ester diacetate, m.p.  $106-108^{\circ}$ ,  $[\alpha]_D^{22} = +134.9^{\circ}$  (chloroform), identical in all respects with the product obtained by independent methods

*Some Chemical Properties of 12-Keto Group of Cholanic Acids, with and without 11-Hydroxy Group*—With the hope that a separation of the 11-

keto compounds from the 12-keto isomers might be achieved through ketonic derivatives, several preliminary experiments were undertaken. 1.0 gm. of methyl 3( $\alpha$ )-acetoxy-12-ketocholanate was heated under a reflux with 1.3 gm. of Girard's Reagent T in 20 ml. of absolute ethanol containing 1.0 ml. of glacial acetic acid. After separation into ketonic and non-ketonic fractions in the usual fashion, 355 mg. were obtained in the non-ketonic fraction. When methyl 3( $\alpha$ ),11( $\beta$ )-diacetoxy-12-ketocholanate and methyl 3( $\alpha$ ),11( $\alpha$ )-diacetoxy-12-ketocholanate were treated under similar conditions, all of the material was obtained in the non-ketonic

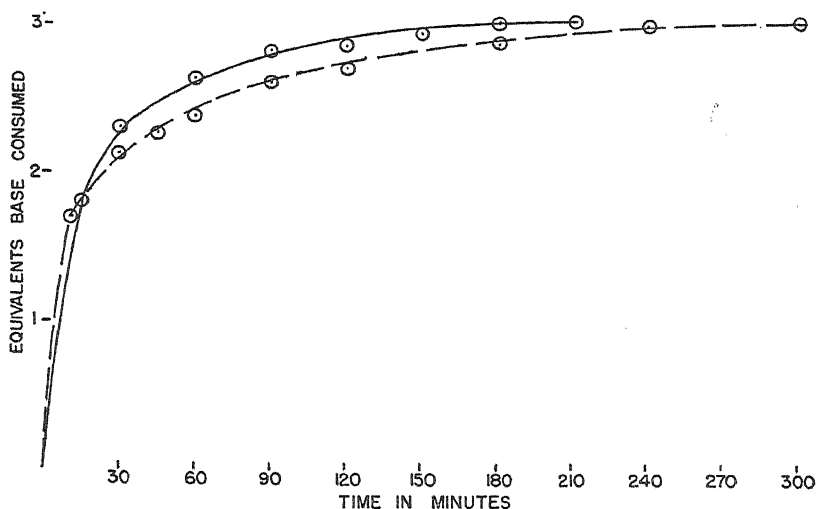


FIG. 3. Alkaline hydrolysis of ketol esters epimeric at C-11. The solid line represents methyl 3( $\alpha$ ),11( $\alpha$ )-diacetoxy-12-ketocholanate; the dash line, methyl 3( $\alpha$ ),11( $\beta$ )-diacetoxy-12-ketocholanate. The constants of the products used were as follows: methyl 3( $\alpha$ ),11( $\alpha$ )-diacetoxy-12-ketocholanate, m.p. 151-152°;  $[\alpha]_D^{25} = +38.5^\circ$  (chloroform); methyl 3( $\alpha$ ),11( $\beta$ )-diacetoxy-12-ketocholanate, m.p. 103-104°;  $[\alpha]_D^{25} = +135^\circ$  (chloroform).

fraction. 3( $\alpha$ ),11( $\alpha$ )-Dihydroxy-12-ketocholanic acid, likewise, did not react with Girard's Reagent T, although this compound forms an oxime (6) but does not form a 2,4-dinitrophenylhydrazone (4). When methyl 3( $\alpha$ ),11( $\alpha$ )-diacetoxy-12-ketocholanate was treated with hydroxylamine under the usual conditions, no oxime was obtained.

*Determination of Relative Rate of Hydrolysis of Methyl Ester Diacetates of Four Isomeric Ring C Ketols*—The experimental results are shown graphically in Figs. 3 and 4. When the amount of material permitted, i.e. with methyl 3( $\alpha$ ),11( $\alpha$ )-diacetoxy-12-ketocholanate and with methyl 3( $\alpha$ ),12( $\beta$ )-diacetoxy-11-ketocholanate, 1 mm was dissolved in 100 ml.



of redistilled 95 per cent ethanol in a 200 ml. volumetric flask. 20 milliequivalents of aqueous sodium hydroxide were added together with water to the mark, while the flask was maintained at 25° in a water bath. Time was measured from the end of the alkali addition. 20 ml. aliquots were removed periodically and transferred into a known excess of 0.1 N sulfuric acid. The titration was then completed with 0.1 N sodium hydroxide to the phenolphthalein end-point. The two less abundant isomers were treated in identical fashion, except that 0.25 mm of the ester was dissolved in a final volume of 50 ml. and 5 ml. aliquots were removed for titration.

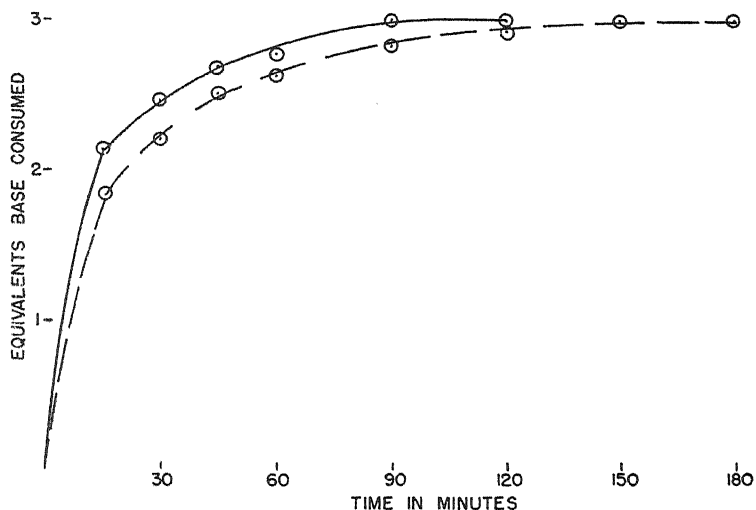


FIG. 4. Alkaline hydrolysis of ketol esters epimeric at C-12. The solid line represents methyl 3( $\alpha$ ),12( $\beta$ )-diacetoxo-11-ketocholanoate; the dash line, methyl 3( $\alpha$ ),12( $\alpha$ )-diacetoxo-11-ketocholanoate. The constants of the products used were as follows: methyl 3( $\alpha$ ),12( $\beta$ )-diacetoxo-11-ketocholanoate, m.p. 83–84°;  $[\alpha]_D^{25} = +55.4^\circ$  (chloroform); methyl 3( $\alpha$ ),12( $\alpha$ )-diacetoxo-11-ketocholanoate, m.p. 133–134°;  $[\alpha]_D^{25} = +118^\circ$  (chloroform).

Only one determination was made with methyl 3( $\alpha$ ),12( $\alpha$ )-diacetoxo-11-ketocholanoate; duplicate determinations were made on the other three products and excellent checks were obtained.

*Methyl 3( $\alpha$ ),12( $\beta$ )-Di-*p*-toluenesulfonyl-11-ketocholanoate*—0.531 gm. of methyl 3( $\alpha$ ),12( $\beta$ )-dihydroxy-11-ketocholanoate, m.p. 156–157°, was dissolved in 2.0 ml. of anhydrous pyridine and cooled in an ice-salt mixture. 1.4 gm. of *p*-toluenesulfonyl chloride and 4 ml. of pyridine were added and the mixture stored at room temperature for 18 hours. After addition of ice to the chilled reaction mixture, the product was extracted with ether, washed with dilute acid, dilute base, and water, and then dried over so-

dium sulfate. 842 mg. of product were obtained which after one recrystallization melted at 154–154.5° with decomposition at 157°. Three further recrystallizations did not change the melting point.  $[\alpha]_D^{22} = +93^\circ$  (acetone).

$C_{33}H_{52}O_9S_2$ . Calculated, C 64.26, H 7.19; found, C 64.26, H 7.21

86.5 mg. were heated with 6 ml. of 1 N NaOH in 50 per cent ethanol under a reflux for 2 hours. The saponification equivalent calculated was 243; found, 243.

*Reaction of Methyl 3( $\alpha$ ),12( $\beta$ )-Di-*p*-toluenesulfonyl-11-ketocholanate with Sodium Iodide*—834 mg. of methyl 3( $\alpha$ ),12( $\beta$ )-di-*p*-toluenesulfonyl-11-ketocholanate together with 1 gm. of sodium iodide were heated under a reflux for 16 hours in acetone. After the 1st hour, sodium *p*-toluenesulfonate crystallized from the solution. The amber solution was filtered, concentrated to dryness under a stream of nitrogen, and dissolved in ether. The ether solution was washed with 1 per cent sodium thiosulfate solution and then with water and dried over sodium sulfate. The solvent was removed and the residue dissolved in 15 ml. of glacial acetic acid. 5.0 gm. of granular zinc were added and the reaction mixture heated for 2 hours under a reflux. The solution was cooled, diluted with ether, and filtered. The ether solution was washed with sodium carbonate and with water and dried over sodium sulfate. After removal of the solvent, the residue failed to crystallize. The product was saponified by heating under a reflux for 2 hours with 16 ml. of 0.25 N sodium hydroxide. The acid obtained by extraction with ether melted at 146–154° and was not further characterized. After esterification with diazomethane, the oily crystals (395 mg.) were dissolved in 25 ml. of petroleum ether and chromatographed over 10 gm. of alumina. 249 mg. of product, melting at 72–74° after crystallization from methanol, were obtained. Two recrystallizations raised the melting point to 75–77°;  $[\alpha]_D = +59.7^\circ$  (methanol);  $[\alpha]_D = +64^\circ$  (acetone).

$C_{25}H_{40}O_4$ . Calculated, C 74.21, H 9.96; found, C 74.39, H 9.76

This is undoubtedly the same compound as the “ $\alpha$ ” form of methyl 11-keto-12-hydroxycholanate or methyl 11-hydroxy-12-ketocholanate of Barnett and Reichstein, for which the melting point 74–76° and  $[\alpha]_D = +54.6^\circ$  (methanol) are recorded (7).

Repetition of this reaction in higher boiling ketones showed that the rate increased with temperature but there was never any indication that the C-12 *p*-toluenesulfonyl group was eliminated. With methyl ethyl ketone (b.p. 81°), 1 equivalent of sodium *p*-toluenesulfonate was obtained after 2 hours heating; with acetonylacetone (b.p. 192°), 1 equivalent was

obtained after 10 minutes. As the reaction temperature increased, more free iodine was liberated.

*Methyl 3( $\alpha$ ),12( $\beta$ )-Dimethanesulfonyl-11-ketocholanate*—2.0 ml. of freshly redistilled methanesulfonyl chloride were added dropwise to 1.0 gm. of methyl 3( $\alpha$ ),12( $\beta$ )-dihydroxy-11-ketocholanate, dissolved in 10 ml. of dry pyridine. After standing for 13 hours at room temperature, the product was isolated in the same manner as the di-*p*-toluenesulfonyl derivative. 1.24 gm. were obtained, m.p. 147–149°. Four recrystallizations from acetone-methanol raised the melting point to 157–158° with decomposition at 161°. It crystallized from acetone as tiny iridescent plates and from acetone-methanol as rosettes of long, fine needles; the melting point of both crystal forms was the same;  $[\alpha]_D^{27} = +55.6^\circ$  (chloroform).

$C_{27}H_{44}O_9S_2$ . Calculated, C 56.22, H 7.69, S 11.11; found, C 56.03, H 7.97, S 11.11

*Reaction of Methyl 3( $\alpha$ ),12( $\beta$ )-Dimethanesulfonyl-11-ketocholanate with Sodium Iodide in Ketone Solutions*—8.6 gm. of the compound were heated under a reflux for 2 hours with 54 ml. of methyl ethyl ketone and 9.15 gm. of sodium iodide. 2.26 gm. of sodium salt were isolated from the reaction. This amount corresponds to the removal of 96 per cent of one methanesulfonyl group, since the sodium methanesulfonate crystallizes as a molecular compound with sodium iodide in the proportion of 4:1. The reaction product was treated as in the previous experiments and after reduction with zinc dust in acetic acid failed to crystallize even after chromatographic separation. When the dimethanesulfonyl compound was treated with sodium iodide in higher boiling ketonic solvents, the products were highly colored and no crystalline derivative was obtained.

Model experiments with methyl 3( $\alpha$ )-acetoxy-12( $\alpha$ )-methanesulfonylcholanate, prepared in amorphous state from methyl 3( $\alpha$ )-acetoxy-12( $\alpha$ )-hydroxycholanate by the procedure previously described and purified by chromatography, indicated that the reaction at the 12 position was especially sluggish, and when vigorous conditions were employed, destruction of the product ensued. Upon heating with sodium iodide for several hours in methyl ethyl ketone, no insoluble salt was formed; when the reaction was conducted in acetonylacetone, the amount of salt obtained indicated the replacement of 72 per cent of the methanesulfonyl group, but no crystalline substance could be isolated from the deep red reaction product after reduction with zinc.

*3( $\alpha$ ),12( $\beta$ )-Bis(hemisuccinoxy)-11-ketocholanic Acid Trimethyl Ester and 3( $\alpha$ )-Hemisuccinoxy-12( $\beta$ )-hydroxy-11-ketocholanic Acid Dimethyl Ester*—When methyl 3( $\alpha$ ),12( $\beta$ )-dihydroxy-11-ketocholanate was heated under a reflux in pyridine with succinic anhydride according to the procedure used

by Schwenk, Riegel, Moffett, and Stahl (8), the product was almost exclusively the disuccinate which was separated as the non-crystalline trimethyl ester by chromatographic adsorption on alumina. 150.9 mg. of the product were saponified in boiling alkali and 11.66 ml. of 0.1 N sodium hydroxide were consumed; calculated for 3( $\alpha$ ),12( $\beta$ )-bishemisuccinoxy-11-ketocholanic acid trimethyl ester, 11.65 ml.; calculated for 3( $\alpha$ )-hemisuccinoxy-12( $\beta$ )-hydroxy-11-ketocholanic acid dimethyl ester, 8.46 ml. The monosuccinate was best prepared from the free acid. 1.50 gm. of 3( $\alpha$ ),12( $\beta$ )-dihydroxy-11-ketocholanic acid were dissolved in 5 ml. of dry pyridine. 3.8 gm. of succinic anhydride in 15 ml. of pyridine were added and the mixture was heated in a water bath at 90° for 40 minutes. It was allowed to cool to room temperature and to stand for an additional 24 hours. The excess succinic anhydride was destroyed with ice and after  $\frac{1}{2}$  hour at room temperature the reaction mixture was extracted with ether. The ethereal solution was washed with dilute acid and with water until neutral. The product crystallized from ethyl acetate. In the event of an incomplete reaction, the ester can be separated from unchanged acid by extraction of the product with acetone in which the succinate is readily soluble in contrast to the insolubility of the original acid. The compound was not characterized at this point but was esterified with diazomethane, and 1.7 gm. of 3( $\alpha$ )-hemisuccinoxy-12( $\beta$ )-hydroxy-11-ketocholanic acid dimethyl ester, melting at 88–89° (9), were obtained after crystallization from ethyl acetate-petroleum ether. Recrystallization from methanol raised the melting point to 90–91°;  $[\alpha]_D^{25} = +63^\circ$  (chloroform).

$C_{30}H_{46}O_8$ . Calculated, C 67.38, H 8.67; found, C 67.20, H 8.64

*3( $\alpha$ )-Hemisuccinoxy-11-keto-12( $\alpha$ )-bromocholanic Acid Dimethyl Ester*—1.2 gm. of 3( $\alpha$ )-hemisuccinoxy-12( $\beta$ )-hydroxy-11-ketocholanic acid dimethyl ester were dissolved in 12 ml. of chloroform which had been freshly redistilled from calcium chloride. The solution was chilled to 0° and 0.4 ml. of freshly redistilled phosphorus tribromide was added. The flask was stoppered and taped, since sufficient pressure is developed in the course of the reaction to dislodge the stopper. After  $\frac{1}{2}$  hour a faint brown color developed. The solution was allowed to stand at room temperature for 24 hours and was then chilled to 0° and 15 ml. of cold saturated sodium bicarbonate solution were added. After  $\frac{1}{2}$  hour at 0°, during which time the flask was shaken intermittently, the chloroform layer was separated. The bicarbonate solution was extracted twice with fresh 50 ml. portions of chloroform and the combined chloroform fractions were washed four times with water. The cloudy chloroform solution was diluted with ether, dried over sodium sulfate, filtered, and concentrated on a steam

bath. The residual oil crystallized from methanol and melted at 79–80°. After recrystallization the melting point was 80–81°;  $[\alpha]_D^{25} = 0^\circ$  (chloroform).

$C_{30}H_{45}O_7Br$ . Calculated, C 60.29, H 7.59, Br 13.37; found, C 59.61, H 7.69, Br 13.72

This reaction was carried out in chloroform and in ether solution at room temperature for 22 hours and in benzene solution at 60° for 2 hours, followed by an additional 20 hours at room temperature. The yield varied from 50 to 70 per cent of the calculated amount. Increasing the time interval to 2 days had no effect upon the yield, nor did the addition of small amounts of solvent saturated with hydrogen bromide. When the reaction was carried out at 0° in chloroform in the presence of solid calcium carbonate, only starting material was recovered.

*3(α)-Hemisuccinoxy-11-ketocholanic Acid Dimethyl Ester*—505 mg. of 3(α)-hemisuccinoxy-11-keto-12(α)-bromocholanic acid dimethyl ester were dissolved in 10 ml. of glacial acetic acid and heated for 1 hour with 500 mg. of zinc dust under a reflux. After cooling, the solution was filtered, diluted with ether, and washed free of acid. The product was esterified with diazomethane and upon removal of the solvent 427 mg. of crystalline product were obtained. After recrystallization from ethyl acetate, 399 mg., m.p. 112–113°, were obtained and two additional recrystallizations from the same solvent raised the melting point to 113–114°;  $[\alpha]_D^{25} = +63^\circ$  (chloroform). The melting point of a mixture with 3(α)-hemisuccinoxy-12(β)-hydroxy-11-ketocholanic acid dimethyl ester was 88–95°.

The compound was characterized by saponification and conversion to the free acid, which after crystallization from methanol and from ethanol melted at 221–224°;  $[\alpha]_D = +67^\circ$  (absolute ethanol). There was no depression of the melting point when this product was mixed with an authentic sample prepared by an independent synthesis. The methyl ester prepared with diazomethane melted at 100–103° and likewise showed no depression on mixture with an authentic sample. Oxidation of the methyl ester with chromium trioxide yielded methyl-3,11-diketocholanoate, m.p. 82–83°, identical with an authentic sample, and showed no depression of the melting point upon admixture.

When the pure bromo keto ester was reduced according to the procedure of Julian *et al.* (10), with the chromous chloride reagent of Conant and Cutter (11), essentially similar results were obtained both as to yield and product. However, when the crude oily bromo keto ester was reduced by this procedure, the products contained considerable chromium and were extremely difficult to purify, whereas reduction of similar products with zinc presented no particular difficulty.

## DISCUSSION

From the products obtained when 3( $\alpha$ ),12( $\beta$ )-dihydroxy-11-ketocholanic acid was isomerized in boiling alkali (Table I), it is immediately apparent that the ketone group at C-11 is the more favored structure, a finding consistent with the known inertness of that ketonic function. The hydroxyl group likewise tends to occupy the more stable configuration and for the 2 carbon atoms C-11 and C-12 this relatively unhindered configuration is not the same. At C-11, the hydroxyl is almost exclusively in the  $\alpha$  configuration, which has been proved to be more stable and subject to less hindrance than the 11( $\beta$ ) configuration. The 12-hydroxyl group, on the other hand, is more stably situated when it is in the  $\beta$  configuration, not only in the ketol but also when the hydroxyl group is the only Ring C substituent. These conclusions are verified by comparison of the rate of saponification of the acetoxy group in the two pairs of ketol esters, diastereoisomeric at C-11 and C-12. As can be seen in Figs. 3 and 4, the 11( $\alpha$ )-acetoxy group is more readily saponified than the 11( $\beta$ )-acetoxy compound; the 12( $\beta$ )-acetoxy substituent is more readily saponified than its 12( $\alpha$ )-diastereoisomer. Despite the activating influence of the  $\alpha$ -keto group in each of the four compounds studied, the resistance toward saponification of the 11-hydroxyl group is clearly evident. Under the same conditions, complete saponification of either 12-acetoxy group was accomplished more quickly than with the 11( $\alpha$ )-acetoxy group and in considerably shorter time than was required for the 11( $\beta$ )-acetoxy group. Thus the rates of saponification as well as the relative proportion of the isomers obtained after alkaline rearrangement are consistent with the structures assigned to the four diastereoisomers.

The readier removal of the C-12 ( $\beta$ )-acetoxy group by alkaline saponification in the two diastereoisomeric ketols is consistent with the results reported for the analogous compound derived from desoxycholic acid (12). In both the ketols and the epimers of desoxycholic acid, it is difficult to establish which groups within the molecule are responsible for the observed differences between the two epimers. While the methyl group at C-13 must be the most significant factor, the side chain is involved, since the acetoxy group at C-12 in the etio acid is more readily hydrolyzed than when the long side chain is intact. Although activation by the  $\alpha$ -carboxyl group of the etio acid is certainly responsible in large part for the increased velocity of saponification of the 12-acetate, it is reasonable that the side chain of the cholanic acid contributes a certain element of hindrance and that this is more effective for the 12( $\alpha$ ) configuration. A decision cannot be reached on present facts, and, as Koechlin and Reichstein (12) have noted, it would be desirable to compare the properties of epimers with no substituent at C-17 in order to assess properly the contributions of the several factors.

The difference in saponification observed between the two epimeric C-11 hydroxy ketols is almost certainly due to the two angular methyl groups, C-18 and C-19, which mask the  $\beta$  configuration. The interference of these methyl groups likewise accounts for the small amount of the 11( $\beta$ )-hydroxy ketol present in the isomerization mixture, since there would be little tendency for the hydroxyl group to occupy this restricted position in preference to the relatively unhindered 11( $\alpha$ ) configuration.

TABLE II  
*Molecular Rotations of Hydroxy Acids and Derivatives Epimeric at C-12*

Compound	$[\alpha]_D$	Solvent	Bibliographic reference No.	$[M]_D$	$[M]_D^\alpha - [M]_D^\beta$
	<i>degrees</i>				
12( $\alpha$ )-Hydroxycholanolic acid	+43.5	Acetone	(19)	16,400	2,100
12( $\beta$ ) epimer	+37.9	"	(19)	14,300	
Methyl 3-keto-12( $\alpha$ )-acetoxycholanate	+83	"	(20)	37,100	17,400
12( $\beta$ ) epimer	+44.1	"	(12)	19,700	18,400
Methyl 3( $\alpha$ ),12( $\alpha$ )-diacetoxycholanate	+94.4	"	(12)	46,300	
12( $\beta$ ) epimer	+56.8	"	(12)	27,900	5,000
Methyl 3( $\alpha$ ),12( $\alpha$ )-dihydroxycholanate	+55.8	"	(20)	22,700	
12( $\beta$ ) epimer	+43.6	"	(12)	17,700	3,600
3( $\alpha$ ),12( $\alpha$ )-Dihydroxycholanolic acid	+47.7	Dioxane	(20)	18,700	
12( $\beta$ ) epimer	+38.4	"	(12)	15,100	23,700
Methyl 3( $\alpha$ ),12( $\alpha$ )-dihydroxyetiocholanate	+106.5	Methanol	(12)	37,300	
12( $\beta$ ) epimer	+38.9	"	(12)	13,600	31,600
Methyl 3( $\alpha$ ),12( $\alpha$ )-diacetoxyl1-ketocholanate	+118.1	Chloroform		59,600	
12( $\beta$ ) epimer	+55.4	"		28,000	

In Table II, the specific and molecular rotations of various compounds bearing substituents at C-12 are compared. It is apparent that in all instances the 12( $\alpha$ ) compound is characterized by the higher rotation and the magnitude of the difference is greatly accentuated by the presence of another functional group in the immediate neighborhood (vicinal action). The exaltation in molecular rotation is greater when a small distance separates the two groups and is best illustrated by comparing the C-12 epimers of the methyl esters of desoxycholic and etiodesoxycholic acids. The cholanolic esters differ by 5000, while the etio esters differ by 23,700. Despite this considerable quantitative effect, the direction is unaltered and it can therefore be assumed that, while vicinal action greatly exag-

gerates the molecular rotation difference, it is without influence upon the direction. The difference of 31,600 in molecular rotation of the two ketols epimeric at C-12 is consistent with this interpretation and is in agreement with the other evidence bearing upon the configurations of the two epimers. This generalization may be extended to the diastereoisomers at C-11 (Table III) where in the ketol the  $\beta$  configuration at C-11 is characterized by the greatest increment, 48,700, when compared to the 11( $\alpha$ ) compound. The epimeric 11-hydroxy bisnor esters show a difference of 18,200 in molecular rotation in favor of the 11( $\beta$ ) compound, which is in the same direction but greater than the 12,200 difference between the two cholanic acids. It is to be noted that the vicinal

TABLE III  
*Molecular Rotations of Hydroxy Acids and Derivatives Epimeric at C-11*

Compound	$[\alpha]_D$	Solvent	Bibliographic reference No.	$[M]_D$	$[M]_D^\beta - [M]_D^\alpha$
	<i>degrees</i>				
3( $\alpha$ ), 11( $\beta$ )-Dihydroxycholanic acid	+55	Methanol	(15)	21,600	13,000
11( $\alpha$ ) epimer	+22	Ethanol	(21)	8,600	
Methyl 3( $\alpha$ ), 11( $\beta$ )-dihydroxy-cholananate	+49	Acetone	(15)	19,900	12,200
11( $\alpha$ ) epimer	+19	Ethyl acetate	(21)	7,700	
Methyl 3( $\alpha$ ), 11( $\beta$ )-dihydroxy-bisnorcholananate	+38.8	Acetone	(22)	14,700	18,200
11( $\alpha$ ) epimer	-9	Chloroform	(23)	-3,500	
Methyl 3( $\alpha$ ), 11( $\beta$ )-diacetoxyl-12-ketocholananate	+135	"		68,100	48,700
11( $\alpha$ ) epimer	+38.5	"		19,400	

action of the carboxyl group in the bisnor esters of the 11-hydroxy acids, where 4 carbon atoms separate the two functional groups, is similar to but not quite so marked as the C-12 epimers of the etio acid where but 2 carbon atoms separate the substituents.

These considerations do not resolve the difficulties encountered when one attempts by rotational data alone to relate the configuration of the four epimeric trihydroxy acids with two substituents in Ring C described by Gallagher (1) and by Wintersteiner, Moore, and Reinhardt (4). It should be noted that the rotational difference between the trihydroxy acids is relatively small. With the accumulation of further information in these and related series, the discrepancy will doubtless be clarified.

It is worthy of comment that vigorous treatment of the ketol with al-



kali yielded only the four anticipated derivatives of cholanic acid; there was no evidence of the formation of any appreciable amount of product in which the configuration of carbon atom 9 had been inverted. This is in harmony with other evidence and would indicate that the urane structure postulated by Marker *et al.* (13) is sterically unfavored in comparison with the normal trans closure between Rings B and C. Thus Kendall and his colleagues (14, 15) obtained only the cholanic acid from the series of reactions involved in the formation and rupture of the 3,9-epoxide of  $\Delta^{11}$ -cholanic acid, despite the very probable formation of a C-9 carbonium ion in the reaction. These authors likewise obtained only the cholanic acid from the catalytic reduction of  $\Delta^{9,11}$ -lithocholanic acid. The experimental evidence indicates then that a cis closure between Rings B and C is markedly strained and that an alternative structure of the urane derivatives might profitably be considered. In contrast to these results, Marker *et al.* (13) reported that urane-3,11,20-trione, upon boiling with alkali, yielded a mixture from which a portion of the original ketone was recovered unchanged. Isomerization at C-17 always occurs with a 20-ketopregnane derivative under these conditions and it is probable that this epimerization accounted in part for the mixture. It appears unlikely from our results that a cis closure at C-9 could have survived the treatment and even more improbable from the work of Kendall that Clemmensen reduction of 3,11,20-triketourane would have yielded a product with a cis Ring B/C closure. Shoppee (16) has recently reviewed the evidence on Ring B/C linkage and has indicated that there is no necessity to postulate a cis type of closure for any of the naturally occurring steroids.

The configuration of the halogen in the bromo keto ester II, obtained when 3( $\alpha$ )-hemisuccinoxy-11-keto-12( $\beta$ )-hydroxycholanic acid dimethyl ester was treated with  $\text{PBr}_3$ , is assigned as  $\alpha$ , since Gerrard (17) has shown that this reaction is accompanied by inversion. The succinoxy group precluded direct comparison with other known 11-keto 12-bromo acids, but additional support for the configuration is the fact that mild alkaline hydrolysis, under conditions which should not have isomerized the ketol, yielded 3( $\alpha$ ),12( $\beta$ )-dihydroxy-11-ketocholanic acid. Since the replacement of halogen by hydroxyl under these conditions is accompanied by inversion, the configuration of the halogen is highly probable. When the replacement of the C-12 hydroxyl group of I by halogen was attempted with thionyl chloride, only oily products were obtained. This reaction may be of some interest, since retention of configuration is usual with this reagent (18) and the expected 12( $\beta$ )-chloroketone should provide the 12( $\alpha$ )-hydroxy ketol upon hydrolysis. However, when satisfactory results were obtained with  $\text{PBr}_3$ , the use of  $\text{SOCl}_2$  was not further explored.

It is to be noted that, in addition to affording a preparative route to an 11-keto bile acid, these experiments have also shown a relatively simple method for the preparation of 3( $\alpha$ ),11( $\alpha$ )-dihydroxy-12-ketocholanic acid. This compound can be converted to 3( $\alpha$ ),11( $\alpha$ )-dihydroxycholanic acid (6) and thus both principal products of the isomerization reaction, which together constitute about 93 per cent of the mixture, can be converted to C-11 oxygenated steroids. Further investigations toward a more efficient utilization of 3( $\alpha$ ),11( $\alpha$ )-dihydroxy-12-ketocholanic acid have been undertaken.

#### SUMMARY

1. 3( $\alpha$ ),12( $\beta$ )-Dihydroxy-11-ketocholanic acid has been converted to 3( $\alpha$ )-hydroxy-11-ketocholanic acid by partial esterification at C-3 with succinic acid, followed by replacement of the C-12 hydroxyl group with bromine and reduction to a methylene group. Alternative methods for the removal of the C-12 hydroxyl group have been explored.

2. The alkaline isomerization of 3( $\alpha$ ),12( $\beta$ )-dihydroxy-11-ketocholanic acid has been studied and the four possible ketols have been isolated and identified.

3. The rate of saponification of the ketol acetates and their optical rotations are consistent with the configurations assigned to the diastereoisomers.

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# LIMITATIONS OF THE USE OF POTASSIUM HYDROXIDE-POTASSIUM CYANIDE MIXTURES IN MANOMETRIC STUDIES

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(Received for publication, September 22, 1948)

The manometric study of biological oxidations by the "direct method" of Warburg, when carried out in the presence of cyanide, involves the use of KOH-KCN mixtures (Krebs (4)) for the absorption of carbon dioxide. The following study describes the rate of distillation of HCN from the experimental solution under various conditions, and suggests a remedy for the difficulties occasionally encountered (Machlis (2)) with the above mixtures.

## EXPERIMENTAL

Cyanide was determined by the modified method of Liebig (Kolthoff and Sandell (3)). To 1.0 ml. of 0.01 M cyanide solution in a test-tube were added 0.5 ml. of  $1.6 \times 10^{-3}$  M potassium iodide and 0.5 ml. of 3.0 M ammonium hydroxide. The mixture was then titrated with  $2 \times 10^{-3}$  M standard silver nitrate to the first permanent appearance of turbidity. For the determination of cyanide in concentrations of 0.001 M or less, the volumes of potassium iodide and ammonium hydroxide solutions were reduced to 0.3 ml., while the standard silver nitrate solution was diluted to  $4 \times 10^{-4}$  M.

Aliquots of a freshly prepared 2.0 M KCN solution were diluted to the desired concentration by the addition of distilled water either alone or with sufficient dilute nitric acid to bring the pH to 6.7. Cyanide concentrations of the control solutions were then determined by the above method. 2 ml. of solution (pH 6.7) were added to each of six Warburg flasks (conical vessels; approximately 16 ml. volume) containing 0.2 ml. of either a KOH, KCN, or KOH-KCN mixture in the center well, together with a small square of fluted filter paper (Umbreit *et al.* (5)). The flasks were equilibrated for 5 minutes, with shaking, in a 30° bath, following which the stop-cocks were closed, the first cup removed from the bath, and 1 ml. of solution taken from the flask for the immediate determination of cyanide. The remaining vessels were removed from the bath at regular intervals and cyanide determinations were carried out.

Some loss of cyanide is effected during the adjustment of the pH (Fig.

1). An additional loss occurs during the short period in which the cyanide solution is transferred to the manometer flasks, and the flasks equilibrated. The latter loss, together with the subsequent disappearance of cyanide, depends upon the nature of the solution in the center well. The use of KOH alone is clearly unsatisfactory for even short time experiments. The distillation of HCN into 10 per cent KOH follows the same course from a 0.001 M solution as from a 0.01 M solution (Fig. 1). In the presence of 10 per cent KOH the pH of the external solution rises from 7.2 at the end of the equilibration period to 8.2 an hour thereafter. With KCN alone in the center well the pH rises from 6.9 to 7.3 during the same interval. The initial pH is 6.7 in all cases.

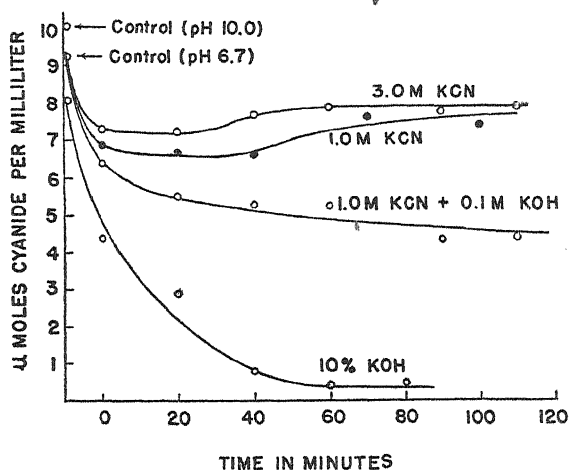


FIG. 1. The effect of  $\text{CO}_2$  absorbents on the disappearance of cyanide from solution. The curve designations represent 0.2 ml. of the specified solution contained in the center well, together with a 4 sq. cm., analytical grade, fluted filter paper.

The concentration of free hydrocyanic acid is 99 per cent of the total cyanide present at pH 7.0 and 87.5 per cent at pH 8.0 (Umbreit *et al.* (5)). Assuming the rate of distillation of HCN to be a function of its concentration, the curve describing the rate of distillation of HCN from a solution buffered at pH 6.7 would, in the presence of 10 per cent KOH, be slightly steeper than the curve presented in Fig. 1, whereas with KCN in the center well little difference would be observed. At pH values of 7.0 and below the rate of distillation will be independent of pH. At higher pH values the rate of distillation will be directly related to the pH.

Equilibrium conditions were examined in systems with KCN exclusively in the center well and no cyanide in the external solutions. Such systems

represented a simplifying approximation to experiments involving very dilute cyanide solutions. When it was desired to simulate the evolution of carbon dioxide by a tissue, 1 ml. of 1.0 M  $\text{HNO}_3$  was added from the side arm to 1 ml. of  $\text{Na}_2\text{CO}_3$  solution (1 mg. of  $\text{Na}_2\text{CO}_3$  per ml.; 212 c.mm. of  $\text{CO}_2$ ) in the main chamber. Otherwise, 2 ml. of either distilled water or phosphate buffer were placed directly in the vessel. The center well in each case contained 0.2 ml. of 2 M KCN together with a fluted filter paper.

Table I indicates that the distillation of cyanide from the center well may be appreciable. The extent of this distillation is directly related to the amount of carbon dioxide absorbed. Equilibrium appears to be achieved within approximately 30 minutes. Neither the pH nor the buffer capacity affects the equilibrium under the experimental conditions employed.

TABLE I

*Rate of Distillation of HCN from Center Well to External Solution*

External solution initially free of cyanide; 0.2 ml. of 2 M KCN and fluted filter paper in the center well.

External solution	Time after equilibration	Cyanide per ml. external solution
	<i>min.</i>	<i>micromoles</i>
Distilled $\text{H}_2\text{O}$ .....	60	2.32
0.5 M $\text{HNO}_3$ .....	60	2.16
0.5 " " + 1 mg. $\text{Na}_2\text{CO}_3$ .....	30	8.80
0.5 " " + 1 " ".....	60	9.52
0.1 " $\text{KH}_2\text{PO}_4$ , pH 7.4.....	60	2.48
0.1 " " " 9.0.....	30	2.20
0.1 " " " 9.0.....	60	2.36

The efficiency with which KCN solutions absorb carbon dioxide was investigated. To this end 0.7 ml. of 4 N  $\text{H}_2\text{SO}_4$  was added from the side arm of a manometer flask to 1 mg. of  $\text{Na}_2\text{CO}_3$  contained in 2 ml. of distilled water. The released carbon dioxide was absorbed as depicted in Fig. 2. Practically, KCN proved as effective as KOH-KCN mixtures in absorbing carbon dioxide.

A tangent was constructed to the curve defined by solid circles (Fig. 2) at a height corresponding to 80 c.mm. of  $\text{CO}_2$  on the ordinate axis. From the slope of this curve at the point of tangency the constant  $C^1$  was derived and, assuming the rate of  $\text{CO}_2$  evolution by the system under study to be 300 c.mm. per hour, the following pertinent data were calculated by the

$^1 X = R/C (1 - e^{-CT})$ , where  $X$  equals the volume of  $\text{CO}_2$  in the flask at any time,  $R$  equals the rate of evolution of  $\text{CO}_2$  by the tissue in c.mm. per minute,  $C$  is a constant, and  $T$  the time in minutes.  $CX$  represents the slope of the curve at any point.

method of Dixon and Elliott (1): (1) the time required to achieve equilibrium (in terms of the  $\text{CO}_2$  concentration in the gas phase), 33 minutes; (2) the concentration of  $\text{CO}_2$  in the gas phase at equilibrium, 36 c.mm.; (3) the error involved by reading the manometer after 10 minutes, 8 c.mm., or less than 3 per cent. For most purposes the error involved

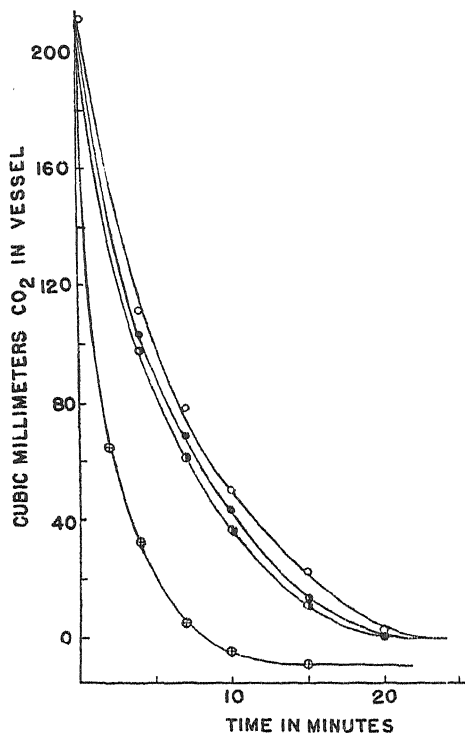


Fig. 2. The absorption of  $\text{CO}_2$  by solutions of potassium cyanide, potassium hydroxide, or mixtures thereof. The curve designations represent the contents of the center well. ○, 1.0 M KCN + Dixon paper; ●, 1.0 M KCN + 0.1 M KOH + fluted paper; ⊙, 1.0 M KCN + 0.1 M KOH + Dixon paper; ⊗, 10 per cent KOH + fluted paper. Dixon KOH paper was made from 4 sq. cm. of analytical grade filter paper rolled into a cylinder with the upper end cut at right angles and spread to offer the maximum surface.

is very small. When the "first 10 minutes" are included in the equilibration period, the error is negligible.

Fluted papers are as effective as the Dixon KOH papers in effecting rapid absorption. Slivers (5 mm.  $\times$  2 cm.) are less satisfactory. The gas uptake in excess of theoretical observed with 10 per cent KOH as absorbent seems to be an oxidation of filter paper as described by Dixon and Elliott.



## DISCUSSION

KOH-KCN mixtures are designed to achieve the same free HCN concentration in the center well as occurs in the external solution. Any addition of KOH beyond the amount necessary for such a condition results in the loss of HCN from the medium (Fig. 1). For most purposes the KCN concentration in the center well is kept at 1.0 M (Umbreit *et al.* (5)), and the KOH concentration is then varied accordingly, the final volume being 0.2 ml.

The proper mixture to be used with 0.001 M cyanide in the external solution demands an alkali concentration of 0.01 M. The acid-binding capacity of 0.2 ml. of 0.01 M KOH is equivalent to but 22.4 c.mm. of carbon dioxide. Since this capacity is soon exceeded by an actively respiring system, it seems desirable to employ KCN exclusively in the center well and thus minimize still further the loss of HCN from the external solution.

The free HCN concentration of a 1.0 M KCN solution, calculated from the hydrolysis constant and the pH of such a solution, is approximately  $5 \times 10^{-3}$  M. Thus only at HCN concentrations less than  $5 \times 10^{-3}$  M in the external medium will any benefit be derived by addition of KOH to the center well. The purpose of KOH in such instances is to prevent a higher HCN concentration in the center well than in the experimental vessel. Since, in most instances, the KOH present is rapidly utilized in the absorption of  $\text{CO}_2$ , the use of KOH-KCN mixture is indicated only in experiments involving dilute ( $10^{-4}$  M or less) cyanide. In the last analysis the choice of a center well solution will depend upon whether it is relatively more hazardous to lose HCN from the external solution to the center well or to exceed the original concentration in the main chamber by distillation of cyanide from the center well. The ability of such mixtures to absorb  $\text{CO}_2$  must be considered as well as the free HCN concentration. The mixtures suggested by Umbreit *et al.* to accompany  $10^{-5}$  M and  $10^{-6}$  M cyanide in the external solution do not have sufficient capacity to make efficient absorbing agents.

## SUMMARY

The rate of distillation of HCN from solution is described during manometric experiments in which either KOH or a KOH-KCN mixture is contained in the center well as an absorbent for  $\text{CO}_2$ . At external cyanide concentrations in excess of  $10^{-4}$  M, the concentration of KOH in a suitable KOH-KCN mixture represents so small a capacity for  $\text{CO}_2$  absorption as to warrant the initial use of KCN alone. At external concentrations of 0.01 M or greater, any KOH in the center well results in a loss of cyanide from solution. At low cyanide concentration, KOH-KCN mixtures in the center well minimize but do not obviate the back-distillation of cy-

anide from the center well to the external solution. KCN is shown to be a satisfactory absorbent for  $\text{CO}_2$ , as indicated by studies of the absorption rates.

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# THE METABOLISM OF CARRIER-FREE RADIOBERYLLIUM IN THE RAT\*

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(Received for publication, September 27, 1948)

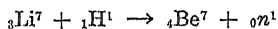
The physiological properties of beryllium and its compounds have assumed considerable significance with the increased economic importance of beryllium in the last 15 years. Early investigators present conflicting reports concerning the metabolism and the toxicity of beryllium (1). More recent studies (2, 3) have shown that the soluble beryllium compounds are highly toxic. The amounts of beryllium needed to kill the average albino rat are, by intravenous injection, 0.4 mg. per kilo, by intraperitoneal injection, 5 to 10 mg. per kilo, and by intratracheal injection, 1 mg. per kilo.

Acute beryllium poisoning manifests itself in at least three organ systems of the body, midzonal necrosis of the liver, necrosis of the distal third of the proximal convoluted tubules of the kidney, and disturbances of the hematopoietic system leading to anemia and leucocytosis.

Due to the necessity of using at least micrograms of material when studies are made with the stable isotope of beryllium, it is difficult to measure the normal metabolism of beryllium because of its toxicity. When a radioactive isotope of this element is used in the absence of stable beryllium, the actual number of beryllium atoms which can be detected is reduced by a factor of at least  $10^6$ . For this reason it is possible to ascertain the manner in which the normal animal handles extremely minute amounts of beryllium which are far below the possible levels of chemical toxicity.

## Method

The  $\text{Be}^7$  used in these studies was produced by the 60 inch Berkeley cyclotron of the Crocker Laboratory by the bombardment of spectroscopically pure lithium metal with 10 m.e.v. protons. The reaction shown below indicates the manner in which this transmutation was effected.



The  $\text{Be}^7$  was isolated from the bombarded lithium by the following procedure. The lithium was dissolved in 50 cc. of water, the solution made

\* This document is based on work performed under contract No. W-7405-eng-48-A-1 for the United States Atomic Energy Commission.

1 N in HCl, and 20 mg. each of copper and zinc carriers were added to aid in the removal of radioactive contaminants, since the lithium was mounted on a copper plate for bombardment. The copper was removed by precipitation with  $\text{H}_2\text{S}$  in the acid solution. 20 mg. of  $\text{FeCl}_3$  were added to the filtrate and then an equal volume of 6 N carbonate-free  $\text{NH}_4\text{OH}$  was added. Under these conditions the  $\text{Be}^7$  was quantitatively coprecipitated with the insoluble  $\text{Fe}(\text{OH})_3$ , which was removed by centrifugation with the zinc remaining in solution. This procedure was repeated three times and the iron removed from the  $\text{Be}^7$  by extraction with isopropyl ether in 6 N HCl. The aqueous fraction contained less than 0.05 mg. of iron and was presumed to be free from appreciable amounts of radioelements other than  $\text{Be}^7$ . The 6 N HCl solution containing the  $\text{Be}^7$  as  $\text{BeCl}_2$  was evaporated almost to dryness, 20 cc. of water were added, and enough carbonate-free NaOH was added to bring the solution to pH 3. Sufficient sodium chloride was added to this solution to make it isotonic. A fraction of the solution was set aside for the decay and absorption studies and the remainder used for the animal experiments.

$\text{Be}^7$  has a half life of 52.9 days<sup>1</sup> and decays solely by orbital electron capture (4, 5). In 10 per cent of the disintegrations the resultant  $\text{Li}^7$  daughter nucleus immediately emits a 0.47 m.e.v.  $\gamma$ -ray (6). The remaining 90 per cent of the disintegrations is not associated with any detectable radiation. The results of the half life measurements made on a sample of the  $\text{Be}^7$ , isolated by the procedure outlined above, are shown in Fig. 1. It will be noted that there are two curves of identical slope. In one, the determinations on the sample were made without a filter being interposed between it and the electroscope; in the second curve the measurements were made on the same sample with a filter which was an aluminum sheet having a mass of 854 mg. per sq. cm. The fact that these two curves not only were identical in slope but also gave a value of a half life of 53 days suggested that the preparation of  $\text{Be}^7$  was essentially free from radioactive contaminants. The greater activity of the sample without the aluminum filter was presumably due to the effect of secondary electrons ejected by the  $\text{Be}^7$   $\gamma$ -rays striking the capsule containing the sample and objects adjacent to the electroscope. Fig. 2 shows the absorption of  $\text{Be}^7$   $\gamma$ -rays by lead. The absorption curve obtained was that for a  $\gamma$ -ray emitter possessing an energy of 0.45 m.e.v., which is in close agreement with the published value of 0.47 m.e.v. (6). The decay curves and  $\gamma$ -ray absorption data indicated that the  $\text{Be}^7$  preparation was sufficiently free from radioactive contamination so as to make it suitable for use in the studies to be described in this article.

A total of fifteen rats was used for these studies. They ranged from 200

<sup>1</sup> Weigand, C., personal communications.

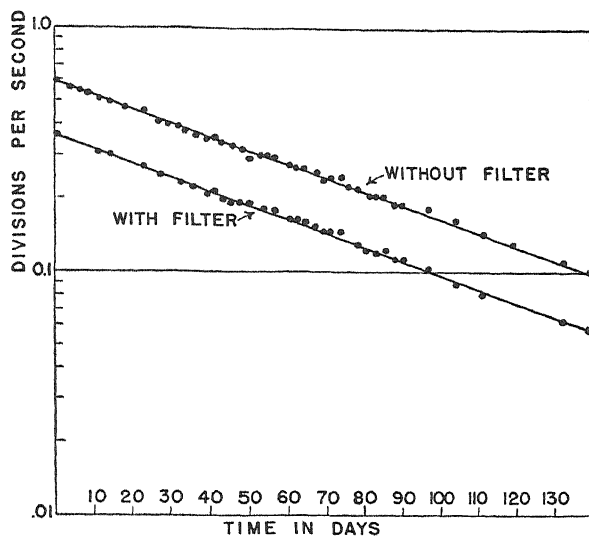


FIG. 1. Decay curve of  $\text{Be}^7$  without filter and with 854 mg. per sq. cm. aluminum filter. Half life, 53 days.

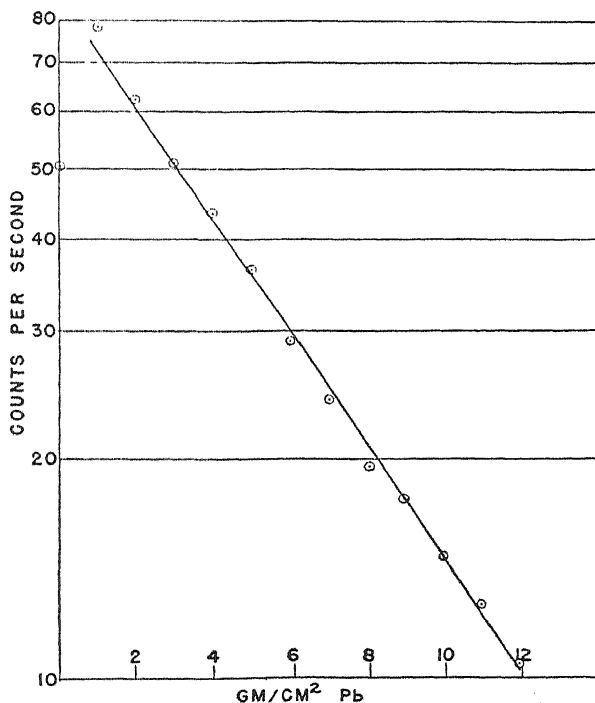


FIG. 2. Mass absorption curve of  $\text{Be}^7$  which is a pure  $\gamma$  emitter. Half value thickness, 3.9 gm. per sq. cm. of Pb. The energy of  $\gamma$ -ray is 0.45 m.e.v.

to 250 gm. in weight. Each of twelve rats received by intramuscular injection 1 cc. of the isotonic solution which contained 1400 counts<sup>2</sup> per second of the Be<sup>7</sup>. The twelve animals were divided into groups of three and each

TABLE I  
*Distribution of Carrier-Free Be<sup>7</sup> in Rat Following Intramuscular Injection*

Tissue	1 day		4 days		16 days		64 days	
	per cent per organ	per cent per gm.	per cent per organ	per cent per gm.	per cent per organ	per cent per gm.	per cent per organ	per cent per gm.
Heart.....	<0.04	<0.04	0.03	0.04	0.04	0.05	<0.06	<0.06
Lungs.....	0.20	0.07	0.15	0.06	0.24	0.08	0.10	0.03
Spleen.....	0.04	0.06	0.09	0.12	0.11	0.16	0.15	0.18
Blood*	0.71	0.05	0.77	0.05	0.81	0.06	0.19	0.01
Liver.....	1.80	0.23	3.68	0.42	2.95	0.27	0.39	0.04
Kidney.....	1.12	0.59	0.75	0.37	0.47	0.25	0.12	0.05
Adrenals.....	<0.04		<0.03		<0.04		<0.06	
Thyroid.....	<0.04		<0.03		<0.04		<0.06	
Lymph gland.....		<0.04		0.06		0.04		<0.06
Pancreas.....	<0.04	<0.04	0.06	0.05	0.05	0.04	<0.06	<0.06
Brain.....	<0.04	<0.04	<0.03	<0.03	<0.04	<0.04	<0.06	<0.06
Fat.....		<0.04		0.03		<0.04		<0.06
Stomach.....	0.06	0.02	0.08	0.02	0.07	0.02	<0.06	<0.06
Small intestine.....	0.40	0.04	0.20	0.02	0.15	0.02	0.09	0.007
Large ".....	0.63	0.08	0.23	0.03	0.19	0.01	0.06	0.006
Skeleton†.....	10.5	0.68	12.3	0.78	16.2	0.87	21.8	1.16
Muscle‡.....	0.67	0.007	0.87	0.008	0.90	0.008	1.59	0.01
Balance.....	1.77		4.35		1.26		0.84	
Left leg.....	56.3		59.5		42.0		20.5	
Skin§.....	0.24	0.008	0.27	0.008	0.23	0.007	0.17	0.005
Pituitary.....	<0.04		<0.03		<0.04		<0.06	
Eyes.....	<0.04	<0.04	0.03	0.08	<0.04	<0.04	<0.06	<0.06
Gonads.....	0.04	0.02	0.06	0.02	0.05	0.02	<0.06	<0.06
Urine.....	15.0		14.6		24.4		44.0	
Feces.....	4.25		4.17		9.25		12.1	
% of dose recovered....	93.7		102.2		99.4		102.1	

\* The value per organ for blood taken as 8 per cent of total body weight.

† Measured value for the entire skeleton.

‡ The value per organ for muscle taken as 45 per cent of total body weight.

§ Animals skinned.

group placed in metabolism cages in order that the urine and feces might be collected separately at daily intervals. A group of three animals was sacrificed at 1, 4, 16, and 64 days after the intramuscular administration

<sup>2</sup> This probability represents approximately 20 microcuries of Be<sup>7</sup>. The explanation for the very low efficiency of measurement appears under "Appendix."

of the Be<sup>7</sup>. An additional group of three animals each received 1 cc. of the Be<sup>7</sup> solution by stomach tube and were sacrificed 8 days later.

The animals were sacrificed by means of chloroform and the tissues taken are listed in Tables I and II. Blood samples were obtained by cardiac

TABLE II

*Distribution of Carrier-Free Be<sup>7</sup> in Rat Following Intramuscular Administration*

The values are given as per cent of dose corrected for recovery and absorption from the injection site.

Tissue	1 day		4 days		16 days		64 days	
	per cent per organ	per cent per gm.	per cent per organ	per cent per gm.	per cent per organ	per cent per gm.	per cent per organ	per cent per gm.
Heart.....	<0.11	<0.11	0.08	0.10	0.01	0.09	<0.08	<0.08
Lungs.....	0.56	0.20	0.39	0.16	0.43	0.13	0.12	0.04
Spleen.....	0.11	0.17	0.23	0.31	0.20	0.29	0.17	0.22
Blood*.....	1.99	0.14	2.01	0.13	1.44	0.11	0.24	0.01
Liver.....	5.04	0.64	9.60	1.09	5.25	0.48	0.48	0.05
Kidney.....	3.14	1.65	1.96	0.96	0.84	0.45	0.15	0.06
Adrenals.....	<0.11		<0.08		<0.07		<0.08	
Thyroid.....	<0.11		<0.08		<0.07		<0.08	
Lymph glands.....		<0.11		0.16		0.07		<0.08
Pancreas.....	<0.11	<0.11	0.16	0.13	0.09	0.07	<0.08	<0.08
Brain.....	<0.11	<0.11	0.08	<0.08	<0.07	<0.07	<0.08	<0.08
Fat.....		<0.11		0.08		<0.07		<0.08
Stomach.....	0.17	0.06	0.21	0.05	0.12	0.04	<0.08	<0.08
Small intestine.....	1.12	0.11	0.52	0.05	0.27	0.04	0.11	0.009
Large ".....	1.76	0.22	0.60	0.08	0.34	0.02	0.08	0.008
Skeleton†.....	29.4	1.90	32.0	2.05	28.8	1.55	27.0	1.44
Muscle‡.....	1.88	0.019	2.25	0.021	1.60	0.014	1.97	0.012
Skin§.....	0.67	0.02	0.70	0.02	0.41	0.01	0.21	0.006
Pituitary.....	<0.11		<0.08		<0.07		<0.08	
Eyes.....	<0.11	<0.11	0.08	0.21	<0.07	<0.07	<0.08	<0.08
Gonads.....	0.11	0.06	0.16	0.05	0.09	0.04	<0.08	<0.08
Urine.....	42.0		38.1		43.5		54.5	
Feces.....	11.9		10.9		16.4		15.0	

\* The value per organ for blood taken as 8 per cent of the total body weight.

† Measured value for the entire skeleton.

‡ The value per organ for muscle taken as 45 per cent of the total body weight.

§ Animals skinned.

puncture. The left hind leg, which was the site of injection, was removed and assayed separately for the fraction of Be<sup>7</sup> that remained unabsorbed. The wet weights of the tissues were determined and then the tissues and excreta were dried at 150° for 2 days and ashed at a temperature of 550° for 24 hours. Preliminary determinations were made by this procedure, and it was demonstrated that no appreciable volatilization of the Be<sup>7</sup> took

place from this method of drying and ashing. The skeleton was separated from the ashed carcass by sifting the loose ash away from the bone by the use of a fine screen. The carcass ash, after separation from the skeleton, is shown in Table I under "balance." The content of  $\text{Be}^7$  in this fraction presumably represents amounts accumulated primarily in the muscle, blood, connective tissue, and fat, since the animals were skinned prior to the ashing of the carcass.

The content of  $\text{Be}^7$  in the samples of rat tissues and excreta was measured with a Geiger counter whose efficiency of detection for  $\gamma$ -rays was of the order of 2 per cent. All of the samples were counted under a lead filter whose mass was 0.99 gm. per sq. cm. This procedure removed the possibility of introducing errors from small amounts of  $\beta$ -emitting contaminants and secondary electrons arising from the samples, the ashing capsules, and surrounding objects. In addition, the lead filter increased the counting efficiency by a factor of almost 2, owing to the ejection of secondary electrons from it.

### *Results*

The distribution of beryllium as carrier-free  $\text{Be}^7$  in the tissues subjected to assay is presented in Table I. The data are expressed in terms of the per cent of uptake of  $\text{Be}^7$  per organ and per gm. of wet weight of the tissue. These values are the averages of the determinations made upon each of the three animals at the appropriate time intervals. The per cent of dose recovered represents the fraction of the administered  $\text{Be}^7$  which could be accounted for by adding together the per cent of uptake in all of that present in the tissues and urine and feces. It may be noted that the over-all recovery in each of the four time intervals was gratifyingly close to 100 per cent.

It may be seen that  $\text{Be}^7$  is only partially absorbed from the injection site, since 56.3, 59.5, 42, and 20.5 per cent of the material administered remained in the left leg at 1, 4, 16, and 64 days respectively after injection. Table II gives the same data as Table I after corrections have been made for the deviations of the recovery from 100 per cent and for the fraction remaining unabsorbed in the injection site. The details of the manner by which these corrections were applied have been fully described in another paper (7).

The results at all four time intervals revealed that the skeleton is the principal organ of accumulation and retention of carrier-free  $\text{Be}^7$ . This element apparently is eliminated from the skeleton very slowly under the conditions of these experiments. This point may be demonstrated if the per cent of  $\text{Be}^7$  absorbed from the injection site is compared to that retained by the skeleton for the same time interval. Thus the ratios of per cent of  $\text{Be}^7$  absorbed to per cent of  $\text{Be}^7$  in the skeleton were found to be 4.2, 3.3, 3.6, and 3.6 at the four time intervals. If the loss from the skeleton



was appreciable during the course of these experiments, the ratios would have become greater than 4 at the later time intervals. It would appear that following absorption a beryllium atom has roughly one chance in three to four of being retained by the skeleton. Obviously this rather crude method of estimation can give no precise information concerning the rate of release of beryllium deposited in the skeleton. However, it serves as an indication that retention of this element in the bone under the conditions of these experiments is quite prolonged.

Considerable accumulation was found to take place in the liver and kidney, but these two organs did not demonstrate to a comparable degree the prolonged retention that was observed to occur in the skeleton. In general, the soft tissue content of  $\text{Be}^7$  was reduced by a factor of approximately 10 during the 64 day time interval of these studies. It is of interest to note that the content of  $\text{Be}^7$  in the spleen and muscle shows no significant decrease during the period of these experiments.

The absorption of  $\text{Be}^7$  from the digestive tract, following administration by stomach tube, could not be demonstrated. On the basis of the amount of  $\text{Be}^7$  given, it was estimated that if any absorption took place it was less than 0.2 per cent of the administered dose.

#### DISCUSSION

The three organs which accumulate and retain beryllium to a marked degree, following the parenteral administration of the radioactive isotope of this element in a carrier-free state, are the skeleton, the liver, and the kidney. The initial concentration of radioberyllium by the liver and kidney is nearly as great as that of the skeleton and much higher than any of the other soft tissues. However, the liver and kidney lose their retained  $\text{Be}^7$  considerably more rapidly than the skeleton. It is of interest to note that the  $\text{Be}^7$  content of the spleen and muscle, while initially considerably less than that of the liver and kidney, remains relatively constant through the entire 64 day interval. The three organs which possess the greatest ability for the accumulation of  $\text{Be}^7$  under the conditions of these experiments are the same structures towards which the toxicological action of stable beryllium is directed when this element is administered parenterally in a soluble form.

It would be of interest to conduct radioautographic studies with radioberyllium in order to ascertain the areas of possible concentration within the histological structures of the bone, liver, and kidney. Since acute beryllium poisoning produces midzonal necrosis of the liver and necrosis of the distal third of the proximal convoluted tubules of the kidney, it can be predicted that these areas should demonstrate the greatest concentration of  $\text{Be}^7$  within the two organs. In the case of bone it seems very possible that the  $\text{Be}^7$  in the carrier-free state would be concentrated in the region of the

osteoid tissue rather than within the mineral structure of the skeleton. These considerations can, of course, only be settled by actual experimental trial with the aid of the radioautographic technique. However, it should be pointed out that the resolution obtained with  $\text{Be}^7$  radioautographs may be less than is usually obtained from radioisotopes which emit electrons. This comes from the fact that  $\gamma$ -rays are very inefficient as compared to electrons in producing darkening of a photographic emulsion, and the secondary electrons produced by the  $\text{Be}^7$   $\gamma$ -rays striking the glass slide of the histological preparation and the backing of the emulsion may render the images indistinct.

### *Appendix*

The presence of small amounts of radioactive contaminants in preparations of  $\text{Be}^7$  can very readily lead to erroneous experimental results.  $\text{Be}^7$  is unique in that the only detectable radiations emitted are  $\gamma$ -rays. The efficiency of counters and electroscopes for the detection of  $\gamma$ -rays is usually of the order of 1 per cent of that for  $\beta$ -particles. Therefore, if only a fraction of a per cent of the number of disintegrations per unit interval of time arises from a radioactive contaminant, the amount of activity detected from the contaminant may equal, or possibly exceed, that produced by the radioberyllium. This, of course, is due to the fact that most artificial radioelements emit  $\beta$ -particles. A very high degree of radiochemical purity of the  $\text{Be}^7$  is obviously essential for tracer studies if the results are to be considered reliable. There are a number of steps which may be taken to reduce the formidable nature of this stringent requirement of radiochemical purity for most tracer applications employing  $\text{Be}^7$ .

1. Counting the samples under a filter as described in the paper will cut out practically all of the  $\beta$ -rays arising from possible radioactive contaminants. For the highest possible efficiency of detection, the filter should be made up of a sheet of aluminum 6 mm. in thickness which is placed directly over the sample, and the top of the aluminum filter should be covered by a lead foil approximately 0.2 mm. in thickness. Such an arrangement will screen out practically all  $\beta$ -rays from radioactive contaminants without significantly attenuating the  $\text{Be}^7$   $\gamma$ -rays, and the superimposed lead foil will provide a maximum number of secondary electrons produced by the  $\gamma$ -rays and thus increase the efficiency of detection by a factor of approximately 2.

2. The employment of spectroscopically pure lithium metal for the cyclotron target will reduce the number and quantity of radioactive contaminants, although this alone will not assure adequate radiochemical purity of the preparation.

3. The cyclotron bombardment should be done with protons rather than deuterons, particularly if the latter particles possess energies in excess of

8 m.e.v. The number of nuclear reactions and the resulting number of radioactive contaminants from impurities in the lithium and the backing material of the target are very much less when protons are used. It is of interest to note that the yield of  $\text{Be}^7$  is almost exactly the same with 20 m.e.v. deuterons and 10 m.e.v. protons, due to the adverse effects of competing nuclear reactions in the bombarded lithium nuclei at the higher energy level of the deuterons.

4. If it is not obligatory to employ carrier-free beryllium in the tracer studies, a very high degree of purification can be attained. This is accomplished by the addition of a few mg. of stable beryllium carrier and the separation of this material from all possible radioactive contaminants by means of the solvent extraction of the basic acetate of beryllium with chloroform (8).

The degree of  $\beta$ -ray contamination can be readily ascertained by determining absorption curves of the preparation. For such determinations the specimen should be spread out in a very thin layer in the center of a sheet of cellophane which is larger than the diameter of the window of either the electroscope or the counter tube. The immediate proximity of any appreciable amounts of solid material to the sample and to either the electroscope or the counter tube should be avoided. These precautions are necessary to reduce the secondary electrons which are ejected in all directions from the  $\text{Be}^7$   $\gamma$ -rays. If these secondary electrons are present in appreciable amounts, they will simulate the presence of  $\beta$ -ray contaminants.

In conclusion it should be pointed out that requirements for radiochemical purity can be reduced considerably by the use of a  $\beta$ -ray filter as described above. However, it should be emphasized that for any attempted radioautographic studies exceedingly clean preparations of  $\text{Be}^7$  are necessary, since a  $\beta$ -ray filter cannot be used and the relative amount of darkening in the photographic emulsion by  $\beta$ -particles is many times greater than the corresponding action of  $\gamma$ -rays.

The authors gratefully acknowledge the assistance of the staff of the 60 inch cyclotron in performing the bombardments for the preparation of the  $\text{Be}^7$ . The advice and suggestions of Professors Edwin M. McMillan and Emilio Segré concerning the problems associated with the nuclear physics and chemistry of  $\text{Be}^7$  were of invaluable assistance to us for both the experimental work and the preparation of this report. We are also indebted to Mrs. Alberta Mozley for technical assistance.

#### SUMMARY

The metabolism of beryllium in the rat has been investigated with tracer amounts of carrier-free  $\text{Be}^7$ . About 20 microcuries of  $\text{Be}^7$  as  $\text{BeCl}_2$  in an isotonic solution of  $\text{NaCl}$  were administered to each rat via intramuscular

injection, and the animals were sacrificed at 1, 4, 16, and 64 days following injection. Within 24 hours 40 per cent of the  $\text{Be}^7$  had been absorbed from the injection site and 54 per cent of the absorbed  $\text{Be}^7$  had been excreted, mainly in the urine. By the 64th day after injection, a total of 80 per cent had been absorbed from the injection site and 70 per cent of the absorbed fraction had been eliminated from the body. Of the absorbed  $\text{Be}^7$  which remained in the body, the major portion was taken up by bone. Within 24 hours after injection the bone had accumulated 29 per cent of the absorbed  $\text{Be}^7$  and maintained this level to the 64th day, when the bone contained 27 per cent of the absorbed material. The initial concentration by the liver and kidney was comparable to that of the skeleton but was followed by more than a 10-fold decrease by the 64th day. The radioberyllium content of the spleen and muscle remained relatively constant throughout the interval of the experiments. Less than 0.2 per cent of the  $\text{Be}^7$  administered orally was absorbed from the digestive tract.

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7. Scott, K. G., Copp, D. H., Axelrod, D. J., and Hamilton, J. G., *J. Biol. Chem.*, **175**, 691 (1948).
8. McMillan, E. M., and Ruben, S., *Phys. Rev.*, **70**, 123 (1946).

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## LETTERS TO THE EDITORS

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### THE IN VITRO INCORPORATION OF THE METHYLENE CARBON ATOM OF GLYCINE INTO RABBIT BONE MARROW FATS

Sirs:

When rabbit bone marrow homogenates were incubated with glycine labeled with  $C^{14}$  in the  $\alpha$ -carbon atom, it was observed that the saturated and unsaturated fatty acids isolated from the lipide fraction of these homogenates contained appreciable  $C^{14}$  activity. These findings are considered of interest in that they might throw light upon the metabolism of bone marrow fats.

The bone marrow homogenate used in these experiments was prepared from femora, tibiae, and humeri of nine rabbits. The bone marrow was removed from the marrow cavities by an air blast and was then homogenized in a Waring blender with 135 ml. of Ringer's solution.<sup>1</sup> The homogenate was divided into three 60 ml. portions, to each of which the following additions were made: 1.21 mm of glycine (containing a  $C^{14}$  activity of  $1.83 \times 10^6$  disintegrations per minute per mm), 0.35 mm of sodium acetate, and 7.5 ml. of 0.05 M phosphate buffer, pH 7.3. The flasks were incubated for varying periods of time at 38° in an atmosphere of 95 per cent  $O_2$  + 5 per cent  $CO_2$ . At the completion of the experiment the material was subjected to high speed centrifugation by a Servall high speed angle centrifuge at about 12,000 R.P.M., which permitted the subsequent removal of the fatty layer at the top of the centrifuge tube. The initial extraction of the fat was carried out as described by Korrodi<sup>2</sup> and the fatty acids then separated into saturated and unsaturated fractions, according to the method described by Hilditch.<sup>3</sup> Both fractions were finally esterified and the methyl esters subjected to  $C^{14}$  analysis by the method of Bale and Masters, as previously described.<sup>4</sup> The results obtained are presented in the accompanying table, expressed in terms of the absolute disintegration rate.

All calculations were made on a mm basis, an average molecular weight

<sup>1</sup> Needham, D. M., Cohen, J. A., and Barrett, A. M., *Biochem. J.*, **41**, 631 (1947).

<sup>2</sup> Korrodi, H., Dissertation, University of Zürich, 1943.

<sup>3</sup> Hilditch, T. P., *The chemical constitution of natural fats*, London, 370-372 (1940).

<sup>4</sup> Altman, K. I., Casarett, G. W., Masters, R. E., Noonan, T. R., and Salomon, K., *J. Biol. Chem.*, **176**, 319 (1948).

of 275 having been assumed for the fatty acid fractions. The data indicate that the methylene carbon atom of glycine is incorporated in the fatty acids of bone marrow and that the isotope concentrations in the saturated fatty acids are considerably higher than in the unsaturated fatty acids.

Time	Saturated fatty acid fraction†	C <sub>0</sub> :C* for saturated fatty acid fraction	Unsaturated fatty acid fraction†	C <sub>0</sub> :C* for unsaturated fatty acid fraction
hrs.				
$\frac{1}{2}$	0.543	337	0.073	2490
$1\frac{1}{2}$	0.322	568	0.119	1530
3	0.615	298	0.127	1440

\* Ratio of isotope concentration of glycine (C<sub>0</sub>) to isotope concentration of the fatty acids (C), on a millimolar basis.

† In units of 10<sup>4</sup> disintegrations per minute per mm.

Since it has been established that acetate serves as a source of carbon for fatty acid synthesis<sup>5</sup> and since Goldinger *et al.*<sup>6</sup> have shown that acetate metabolism plays a highly important rôle in the bone marrow, these experiments suggest that rabbit bone marrow might be capable of effecting the conversion of glycine to acetate, although it has been reported that in the pigeon<sup>7</sup> and in *Clostridium cylindrosporium*<sup>8</sup> glycine and acetate are not interconvertible. Further studies are in progress.

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<sup>5</sup> Rittenberg, D., and Bloch, K., *J. Biol. Chem.*, **154**, 311 (1944); **160**, 417 (1945).

<sup>6</sup> Goldinger, J. M., Lipton, M. A., and Barron, E. S. G., *J. Biol. Chem.*, **171**, 801 (1947).

<sup>7</sup> Buchanan, J. M., Sonne, J. C., and Delluva, A. M., *J. Biol. Chem.*, **173**, 81 (1948).

<sup>8</sup> Barker, H. A., and Elsdon, S. R., *J. Biol. Chem.*, **167**, 619 (1947).

## THE ACTIVATING EFFECT OF *m*-NITROANILINE ON THIAMINE DESTRUCTION BY THE CHASTEK PARALYSIS ENZYME

*Sirs:*

*m*-Aminobenzyl-4-methylthiazolium chloride has been found to activate or increase the destruction of thiamine by the Chastek paralysis enzyme, in contrast to the behavior of the corresponding *o*-amino analogue, which is a strong inhibitor.<sup>1</sup> A number of facts more recently available have suggested that the activation produced by the former compound is due to its combination through its amino group with the 5-methylene group of the pyrimidine moiety as the latter is split from thiamine in the course of the enzyme reaction. A secondary amine is thus formed, and the amount of enzymatic reaction is increased by this addition of artificial "reactor" substance. This hypothesis is in keeping with the finding of Krampitz and Woolley<sup>2</sup> that enzyme *extracts* yield a pyrimidine derivative in the thiamine decomposition, whereas whole tissue suspensions carry the reaction to the more complete stage with release of the pyrimidylmethyl alcohol. The isolation by Hennessy and Warner<sup>3</sup> of a crystalline pyrimidine derivative (after destruction of thiamine by the enzyme obtained from clams) is likewise in agreement.

Other amines such as *m*-nitroaniline and *m*-aminobenzoic acid are also strong activators of the enzymatic reaction.<sup>4</sup> The above hypothesis suggests that it should be possible to isolate the pyrimidine derivative of one of these simpler activators. Consequently, after a preliminary experiment, 1.69 gm. (5.0 millimoles) of thiamine chloride hydrochloride were incubated at pH 7.4 and 37.5° for 4 hours in 5 liters of 0.04 M phosphate buffer and 4 per cent sodium chloride with the soluble portion from 25 gm. of acetone-desiccated enzyme preparation (carp viscera) and 6.9 gm. of *m*-nitroaniline.

At the conclusion of the incubation, analysis disclosed the destruction of 73.3 per cent (3.67 millimoles) of thiamine. The deproteinized solution adjusted to a slightly alkaline reaction was exhaustively extracted with butyl alcohol. Upon subsequent fractionation with benzene, chloroform, and butyl alcohol, the unused *m*-nitroaniline was recovered and, in addition, 0.9 gm. (74.5 per cent yield) of a crystalline dihydrochloro-

<sup>1</sup> Sealock, R. R., and Livermore, A. H., *J. Biol. Chem.*, **177**, 553 (1949).

<sup>2</sup> Krampitz, L. O., and Woolley, D. W., *J. Biol. Chem.*, **152**, 9 (1944).

<sup>3</sup> Hennessy, D. J., and Warner, S., Abstracts, American Chemical Society, 109th meeting, Atlantic City, New Jersey (April, 1946).

<sup>4</sup> Sealock, R. R., and Davis, N. C., unpublished data.

ride melting with decomposition at 211–212° (corrected). The free base, which crystallized on addition of alkali to the acid solution, is yellow and melts at 227.5–228.5° (corrected). These melting points are identical with those obtained with the secondary amine, N-(2-methyl-6-aminopyrimidyl-5-methyl)-*m*-nitroaniline, synthesized from the corresponding pyrimidylmethyl bromide and *m*-nitroaniline by warming the alcoholic solution in the presence of sodium bicarbonate. The mixed melting point showed no depression, and identity was further established by comparison of ultraviolet absorption spectra in aqueous solution at pH 5.0 and 7.0 and in 0.1 N sodium hydroxide (maximum at 2420–2430 Å). All properties were identical with those of the compound isolated in smaller yield in the preliminary experiment. It was therefore concluded that the compound produced by the enzymatic destruction of thiamine in the presence of *m*-nitroaniline is the above pyrimidine derivative, in agreement with the hypothesis.

It also has been concluded that those amines which activate, rather than inhibit, the enzymatic destruction do so by entering into the reaction at a stage which probably follows the formation of the enzyme-substrate complex. They in turn are converted to similar pyrimidylmethyl derivatives. In the above experiment the artificially added "reactor" amine (*m*-nitroaniline) overwhelmed the naturally occurring one which was present in limiting concentration because of the relatively small amount of enzyme intentionally employed. For the purpose of further experimentation it is of interest to bear in mind that the reaction *may represent* an enzymatic transmethylation with a substituted methyl group.

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## VITAMIN B<sub>12</sub> AND RELATED FACTORS IN THE NUTRITION OF LACTOBACILLUS LEICHMANNII 313

Sirs:

The joint occurrence of thymidine and vitamin B<sub>12</sub> in many natural products and their interchangeability in the nutrition of *Lactobacillus leichmannii* (strain 313 and ATCC 4797) complicate the interpretation of the usual tube microbiological assay for either entity.<sup>1</sup>

We have found it possible to separate complex mixtures containing vitamin B<sub>12</sub> and thymidine on paper strip chromatograms, using *n*-butanol as the mobile phase. The positions and hence the identities of the various growth factors on a developed chromatogram are revealed by a general technique reported earlier.<sup>2</sup>

The strip chromatograms are laid on a nutrient agar containing the constituents of a medium used in the titrimetric determination of apparent vitamin B<sub>12</sub> activity. The agar is seeded with *L. leichmannii* 313. After incubation, zones of growth of the organism are seen along the loci of the chromatograms.

Analysis of samples of various preparations known to possess antipernicious anemia or animal protein factor activity has revealed the existence of at least six entities capable of supporting the growth of *L. leichmannii* 313 in the vitamin B<sub>12</sub>-deficient medium. Each entity occupied a characteristic position on a chromatogram.  $R_F$  values, measured in the usual fashion, varied somewhat from day to day. However, on a given day a specific entity had a definite  $R_F$  value independent of the particular preparation in which it was found. The range of  $R_F$  values observed for the six growth factors was as follows: (1) 0.0 to 0.03, (2) 0.03 to 0.10, (3) 0.15 to 0.20, (4) 0.26 to 0.36, (5) 0.41 to 0.54, (6) 0.52 to 0.67. (The  $R_F$  values of the two slowest substances are subject to great error. These substances were not actually completely separated but caused a connected doublet zone of growth.)

Analysis of a concentrate of vitamin B<sub>12</sub> (obtained through the courtesy of Dr. W. L. Sampson of Merck and Company) revealed the characteristic doublet zone of growth due to the two slowest factors referred to above. A similar finding was made in the case of the crystalline antipernicious anemia factor obtained through the courtesy of Dr. T. H. Jukes of the Lederle Laboratories. The two substances present may or may

<sup>1</sup> Hoffmann, C. E., Stokstad, E. L. R., Franklin, A. L., and Jukes, T. H., *J. Biol. Chem.*, **176**, 1465 (1948). Skeggs, H. R., Huff, J. W., Wright, L. D., and Bosshardt, D. K., *J. Biol. Chem.*, **176**, 1459 (1948).

<sup>2</sup> Winsten, W. A., and Eigen, E., *Proc. Soc. Exp. Biol. and Med.*, **67**, 513 (1948).

not represent the two forms of crystalline APA factor reported by Smith.<sup>3</sup>

A preparation of thymidine obtained from Dr. A. L. Franklin of the Lederle Laboratories was found to cause a principal zone of growth ( $R_F = 0.54$ ) and a minor zone with an  $R_F = 0.41$ . It is not possible at this writing to state unequivocally which of these zones of growth is due to thymidine itself or what the other zone represents.

A series of commercial injectable liver preparations and one preparation of animal protein factor concentrate, intended for the treatment of pernicious anemia, were analyzed. All contained the two slowest substances forming the characteristic doublet zone of growth, and all contained one or more of the four alternative growth factors referred to above. The relative concentrations of the six factors varied widely, as indicated qualitatively by the sizes of various zones of growth.<sup>4</sup>

The nature of the three alternative growth factors which replace vitamin B<sub>12</sub> and thymidine remains to be determined. However, account must be taken of their possible presence and influence in any microbiological assay which measures total apparent B<sub>12</sub> activity.

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Received for publication, January 3, 1949

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<sup>3</sup> Smith, E. L., *Nature*, **161**, 638 (1948).

<sup>4</sup> In testing the different preparations, 10  $\mu$ l. samples containing 0.5 to 9.0  $\gamma$  per ml. of apparent vitamin B<sub>12</sub> as measured by titrimetric microbiological assay were chromatographed.

## A SPECIFIC INHIBITOR FOR DESOXYRIBONUCLEASE\*

*Sirs:*

The epithelium of the crop gland of a pigeon undergoes very rapid growth during the latter part of the brooding period. After the young hatch, the hypertrophic epithelium desquamates and constitutes the crop milk.<sup>1</sup> This tissue still retains the cellular structure.<sup>2</sup>

0.1 to 0.4 gm. portions of crop gland tissue were obtained by biopsy during active growth and at rest. The tissue, with 10 volumes of water added, was ground with sand, and the mixture was centrifuged. The water extract was tested for desoxyribonuclease.<sup>3</sup> No activity was found at either stage.

	Per cent activity
Desoxyribonuclease alone.....	100.0
"          +	
0.1 cc. fresh extract, hypertrophic.....	21.4
0.1 " " " normal.....	65.0
1.0 " frozen extract, hypertrophic.....	16.7
1.0 " " " normal.....	14.7
1.0 " boiled extract hypertrophic.....	114.0
1.0 " " " normal.....	98.0
1.0 " extract, incubated with 0.25 $\gamma$ trypsin for 5 hrs.....	80.7
1.0 " " not dialyzed.....	23.0
1.0 " " dialyzed 20 hrs. at 5°.....	28.0
1.0 " 0.3 ppt., volume reduced 5 times	25.4
1.0 " 0.6 " " " 5 " .....	4.4

It then occurred to us that the gland may contain an inhibitor specific for desoxyribonuclease. The test system was composed of thymonucleic acid prepared according to Hammarsten,<sup>4</sup> once recrystallized desoxyribonuclease prepared according to Kunitz,<sup>5</sup> and variable amounts of crop gland extract; other conditions were the same as previously described.<sup>3</sup> Inhibition was found in all experiments. It was more pronounced during the hypertrophic stage.

\* Aided by grants from the Donner Foundation, Inc., the John and Mary R. Markle Foundation, and the United States Public Health Service.

<sup>1</sup> Dabrowska, W., *Comp. rend. Soc. biol.*, **110**, 1091 (1932).

<sup>2</sup> Litwer, G., *Z. wiss. Biol., Z. Zellforsch. u. mikr. Anat.*, **3**, 695 (1926).

<sup>3</sup> Laskowski, M., and Seidel, M. K., *Arch. Biochem.*, **7**, 465 (1945).

<sup>4</sup> Hammarsten, E., *Biochem. Z.*, **144**, 383 (1924).

<sup>5</sup> Kunitz, M., *Science*, **108**, 19 (1948).

Two brooding and two non-brooding pigeons were killed; the crop glands were removed and extracted as before. The extract was frozen in small samples and thawed just before use. The freezing produced a loss in inhibitory power of the extracts from brooding pigeons, but none in the extracts of non-brooding birds.

The inhibitor is a protein. It is destroyed by heat, by exposure to pH 3.0, and by trypsin. It is not lost on dialysis at 5° and is precipitated by ammonium sulfate. The most active fraction is precipitated between 0.3 and 0.6 saturation. A few typical results are presented in the table. Activity was calculated in viscosity units,<sup>3</sup> and is expressed in per cent of activity recovered, assuming the non-inhibited value as 100 per cent.

The presence of the inhibitor was demonstrated in other tissues, including rat liver. The inhibitor described by us is probably not identical with the inhibitor described recently by Zamenhof and Chargaff<sup>6</sup> since the latter was reported to inhibit the depolymerase from yeast, and did not inhibit partially purified pancreatic desoxyribonuclease.

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<sup>6</sup> Zamenhof, S., and Chargaff, E., *Science*, **108**, 628 (1948).

<sup>7</sup> On leave of absence from the Agricultural Institute, Pulawy, Poland.

# THE NON-SPECIFICITY OF THYMIDINE AS A GROWTH FACTOR FOR LACTIC ACID BACTERIA\*

Sirs:

The equivalence of thymidine, refined liver extracts (vitamin B<sub>12</sub> ?), and ascorbic acid in promoting growth of certain bacteria in defined media has stimulated considerable interest.<sup>1, 2</sup>

Further study showed that, for eleven of thirteen organisms, thymidine, hypoxanthine desoxyriboside, adenine desoxyriboside, or cytosine desoxy-

	Amount required to detect response	Amount required for maximum growth	Growth* of				
			<i>L. leichmannii</i> 313	<i>L. acidophilus</i> 832	<i>L. acidophilus</i> 4913	<i>L. delbrueckii</i> 730	<i>Leuconostoc citrovorum</i>
No supplement	—	—	—	—	—	—	—
Thymine desoxyriboside	2 γ	20 γ	+++	+++	+++	+++	++
Adenine desoxyriboside	2 “	20 “	+++	+++	+++	±	—
Hypoxanthine desoxyriboside	2 “	20 “	+++	+++	+++	±	—
Cytosine desoxyriboside	2 “	20 “	+++	+++	+++	±	—
Desoxyribonucleic acid	20 γ	2-500 γ	+++	±†	—	+++	—
Ascorbic acid	3 mg.	50 mg.	+++	—	+++	—	—
Reticulogen	0.1 μl.	5 μl.	+++	++	++	—	+++

—, no growth; ±, slight growth; ++, good growth; +++, heavy growth.

\* 24 hours incubation at 37° in 10 ml. of a previously described medium.<sup>1</sup>

† Heavy growth obtained with 300 mg. of DNA in 48 hours.

riboside was equally active in promoting growth. Refined liver extract and desoxyribonucleic acid (DNA) were also active for many organisms. Ascorbic acid was active only rarely and at much higher concentrations. Representative types of behavior are illustrated in the table. *Lactobacillus delbrueckii* 730 is unique among the organisms tested in having a specific requirement for thymidine which is not adequately replaced by the other desoxyribonucleosides or by low levels of liver extract.

If the desoxyribonucleosides function by permitting synthesis of DNA, then in most of these organisms this is permitted by either (a) the active

\* Supported in part by a grant from the United States Public Health Service. We are indebted to the P. A. Levene collection and to Dr. T. G. Brady and Dr. J. O. Lampen for samples of various desoxyribonucleosides.

<sup>1</sup> Snell, E. E., Kitay, E., and McNutt, W. S., *J. Biol. Chem.*, **175**, 473 (1948).

<sup>2</sup> Skeggs, H. R., Huff, J. W., Wright, L. D., and Bosshardt, D. K., *J. Biol. Chem.*, **176**, 1459 (1948). Hoffmann, C. E., Stokstad, E. L. R., Franklin, A. L., and Jukes, T. H., *J. Biol. Chem.*, **176**, 1465 (1948). Shive, W., Ravel, J. M., and Eakin, R. E., *J. Am. Chem. Soc.*, **70**, 2614 (1948).

factor of liver extract (presumably vitamin B<sub>12</sub>) or (b) any one of four desoxyribonucleosides. It is possible that the desoxyribonucleosides function interchangeably by supplying their common portion, either as desoxyribose or as desoxyribose phosphate, for synthesis of DNA, and that this common portion may be formed by independent processes involving vitamin B<sub>12</sub>. However, a hydrolysate of guanine desoxyriboside prepared to liberate free desoxyribose<sup>3</sup> proved inactive. Further work is thus required to explain the complex interrelationships indicated by these common effects upon growth.

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<sup>3</sup> Levene, P. A., and Bass, L. W., Nucleic acids, American Chemical Society monograph series, New York, 182 (1931).

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